STUDIES OF THE HUMAN LEUCOCYTE DIFFERENTIATION ANTIGEN CDw75.

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To my husband Mark, and
the memory of our son
Andrew Coldwell.

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DECLARATION.

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ABSTRACT.

Using monoclonal antibodies (mAbs) characterised at the Fourth International Leucocyte Typing Workshop, the expression and distribution of the cell-surface antigen CDw75 on normal peripheral blood lymphocytes and on cells from patients with B-cell chronic lymphocytic leukaemia (B-CLL) was studied and further defined. In addition, expression of the antigen on solid lymphoid and non-lymphoid tissues was studied, and a comparison was made between expression on normal tissues and on tumour tissues.

The structure of the epitopes recognised by the different CDw75 mAbs was also analysed, and several attempts were made to isolate and characterise the antigen biochemically. The CDw75 antigen had been reported to be a cell-surface β-galactoside-α-2,6-sialyltransferase enzyme, the amino acid sequence of which had already been determined. From this amino acid sequence, synthetic peptides were constructed and mAbs were raised against sialyltransferase. These mAbs were used to isolate the enzyme and characterise it biochemically.

The results of this study show that CDw75 is expressed primarily on the surface of mature B-cells and on leukaemias and lymphomas. It is also expressed on T-cell subsets, and different epitopes are expressed on T-cells at different activation states. CDw75 is also expressed in B-cell areas of lymphoid tissues as well as on non-lymphoid tissues such as liver, breast, pancreas and intestine. Expression levels are altered in tumour tissues.

The CDw75 mAbs do not recognise a single epitope. Some recognise a sialylated epitope, whereas one epitope is masked by sialic acid. The α-2,6-sialyltransferase enzyme is expressed in a perinuclear area of the cell, likely to be the Golgi Apparatus, and is not expressed on the surface of B-cells. The CDw75 antigen is not a cell-surface sialyltransferase enzyme, but is a cell-surface carbohydrate epitope whose expression is regulated by the activity of the β-galactoside-α-2,6-sialyltransferase enzyme.

ABBREVIATIONS.

B-CLL	B-cell chronic lymphocytic leukaemia
BSA	Bovine serum albumin
CAMs	Cell adhesion molecules
CMPNeuAc	Cytidine monophosphate Neuraminic Acid
ELISA	Enzyme linked inmmunosorbent assay
FACScan	Fluorescence activated cell scanner
FCS	Foetal calf serum
FITC	Fluorescein Isothiocyanate
Galβ1,4GlcNAc	β1,4-Galactose-N-Acetyl Glucosamine
GlcNAc	N-Acetyl glucosamine
HRP	Horse radish peroxidase
Ig	immunoglobulin
kDa	kilo Daltons
MAA	Maackia Amurensis Agglutinin
mAb	monoclonal antibody
MPC	Magnetic particle collector
m_r	molecular weight
N-CAMs	Neural cell adhesion molecules
NHSS-biotin	sulpho-N-hydroxysuccinimidobiotin
NK cells	Natural killer cells
NRS	Normal rabbit serum
PBL	peripheral blood lymphocytes
PBS	Phosphate buffered saline
PE	Phycoerythrin
PEG	Polyethylene glycol
PHA	Phytohaemagglutinin
SDS PAGE	Sodium dodecyl sulphate polyacrylamide gel electrophoresis
sIg^+	surface immunoglobulin positive
SNA	Sambucus Nigra Agglutinin
TBS	Tris buffered saline
v/v	volume/volume

CONTENTS.

CHAPTER 1

Introduction.

1.1 The Human Leucocyte Differentiation Antigen: CDw75.

CDw75 is a cell-surface antigen found predominantly on cells of B-cell origin, but is also expressed on some T-cell subsets(Dorken, B., Moller, P., Pezzutto, A., Schwartz-Albiez, R. and Moldenhauer, G., 1989). It was originally clustered in 1989 at the Fourth International Leucocyte Typing Workshop where four monoclonal antibodies (mAbs) were designated to be part of this cluster. These are HH2(Smeland, E., Funderud, S., Ruud, E., Blomhoff, H.K. and T, G., 1985), OKB4(Mittler, R.S., Talle, M.A., Carpenter, K., Rao, P.E. and Goldstein, G., 1983), LN-1(Epstein, A.L., Marder, R.J., Winter, J.N. and Fox, R.I., 1984), and EBU-141(Gramatzki, M., Lauer, U., Burger, R., Huber, C., Rohwer, P., Kalden, J.R. and Henschke, F., 1989), with a fifth mAb, EBU-65 also found to exhibit "CDw75-like" staining patterns(Gramatzki, M., Burger, R., Kraus, J., Lauer, U., Rohwer, P., Eger, G., Kalden, J.R. and Henschke, F., 1991). It has since been shown that it is statistically likely that this mAb also recognises CDw75(Guy, K. and Andrew, J.M., 1991). Interestingly, all five mAbs are of IgM isotype, and although OKB4 was first cloned in 1983, LN-1 in 1984, HH2 in 1985 and EBU-141 and EBU-65 in 1989, at the start of this project in 1990, very little was known about the structure of the antigen CDw75 which is identified by all of these mAbs.

Surprisingly, the five mAbs were produced in response to a variety of different immunogens. HH2 was raised in mice in response to immunisations with lymphoma cells from a patient with a follicular lymphoma. OKB4 was produced by immunisation with Raji cells and cells from a patient with Burkitt's Lymphoma. EBU-141 and EBU-65 were both raised in response to immunisations with the plasma cell line U266, and LN-1 was produced by immunising mice with isolated nuclei from pokeweed mitogen-stimulated peripheral blood lymphocytes (PBL).

Previous reports indicated that CDw75 was only expressed on mature surface immunoglobulin positive (sIg^+) B-cells, and corresponding mature B-cell-like leukaemias and lymphomas, but not on Ig-secreting plasma cells, or plasma cell tumours(Dorken, B., Moller, P., Pezzutto, A., Schwartz-Albiez, R. and Moldenhauer, G., 1989). This distribution seems strange if we consider that mAbs EBU-141 and EBU-65 were raised in response to a plasma cell line. The mAbs have also been reported to stain some normal peripheral blood T-cells with low intensity, and in solid lymphoid tissue, all mAbs strongly stain B-cells of germinal centres. They

also weakly stain some cells of the mantle zone(Dorken, B., Moller, P., Pezzutto, A., Schwartz-Albiez, R. and Moldenhauer, G., 1989).

LN-1 was found to stain erythrocyte precursors of the bone marrow, ciliated epithelial cells of the bronchus, distal tubular cells of the kidney, ductal cells from several organs, and most epithelium-derived cell lines(Okon, E., Felder, B., Epstein, A., Lukes, R.J. and Taylor, C.R., 1985; Erikstein, B.K., Beiske, K., Smeland, E.B., Davies, C.D.L., Blomhoff, H.K. and S, F., 1989). The LN-1 epitope is also neuraminidase sensitive, which means that it must contain sialic acid. HH2 is reported to stain the same types of lymphoid cells as LN-1 (Smeland, E., Funderud, S., Ruud, E., Blomhoff, H.K. and T, G., 1985; Erikstein, B.K., Beiske, K., Smeland, E.B., Davies, C.D.L., Blomhoff, H.K. and S, F., 1989). OKB4 reacts with Burkitt's Lymphoma cells and 90% of sIg^+ cells, but does not stain activated B-cells(Mittler, R.S., Talle, M.A., Carpenter, K., Rao, P.E. and Goldstein, G., 1983). EBU-141 and EBU-65 are also reported to bind to mature sIg^+ B-cell-type leukaemias and lymphomas(Gramatzki, M., Lauer, U., Burger, R., Huber, C., Rohwer, P., Kalden, J.R. and Henschke, F., 1989), but also to hepatocytes and glandular structures such as salivary gland ducts, salivary acini, sweat glands, endometrial glands and prostatic gland cells(Gramatzki, M., Burger, R., Kraus, J., Lauer, U., Rohwer, P., Eger, G., Kalden, J.R. and Henschke, F., 1991).

Therefore, the cluster CDw75 defines a cell-surface antigen, predominantly of B-cells, which appears at a stage of differentiation around the same time as sIg, and which is lost from the cell-surface when these cells are further activated to become plasma cells which secrete Ig. Limited information is available as to the tissue distribution of the antigen outside of lymphoid tissue, and no direct comparisons of staining patterns of each of the five mAbs in different tissues has yet been reported. Biochemical information about the antigen is also very sparse. Immunoprecipitation of antigens with OKB4 and EBU-141 have been reported giving molecular weights (m_rs) of 87 kDa (Mittler, R.S., Talle, M.A., Carpenter, K., Rao, P.E. and Goldstein, G., 1983) and 53 kDa(Dorken, B., Moller, P., Pezzutto, A., Schwartz-Albiez, R. and Moldenhauer, G., 1989) with OKB4 and 34-38 kDa with EBU-141(Okon, E., Felder, B., Epstein, A., Lukes, R.J. and Taylor, C.R., 1985). However, no satisfactory evidence for such claims has yet been published.

In 1990, it was reported that the CDw75 gene had been cloned(Stamenkovic, I., Asheim, H.C., Deggerdal, A., Blomhoff, H.K., Smeland, E.B. and Funderud, S.,

1990). A cDNA library was introduced into COS cells and was screened using HH2 to label the cell surface. A positive cDNA clone was reintroduced into COS cells and was found to react with all CDw75 mAbs. The sequence of this cDNA was found to be almost identical to that of the human enzyme β-galactoside-α-2,6-sialyltransferase. It was therefore claimed that CDw75 had been identified as a cell-surface version of this enzyme. It was later shown that transfection of this cDNA into COS cells also leads to expression of the EBU-65 epitope(Erikstein, B.K., Funderud, S., Beiske, K., Aas-Eng, A., De Lange Davies, C., Blomhoff, H.K. and Smeland, E.B., 1992), and that HH2 not only stained the surface of these transfected cells, but also stained the cytoplasm in the juxtanuclear region. This seemed to suggest that not only was EBU-65 definitely part of the CDw75 cluster, but that the sialyltransferase enzyme might be expressed in the Golgi Apparatus, where it is more conventionally found, as well as on the cell surface.

1.2 Human β-galactoside-α-2,6-sialyltransferase.

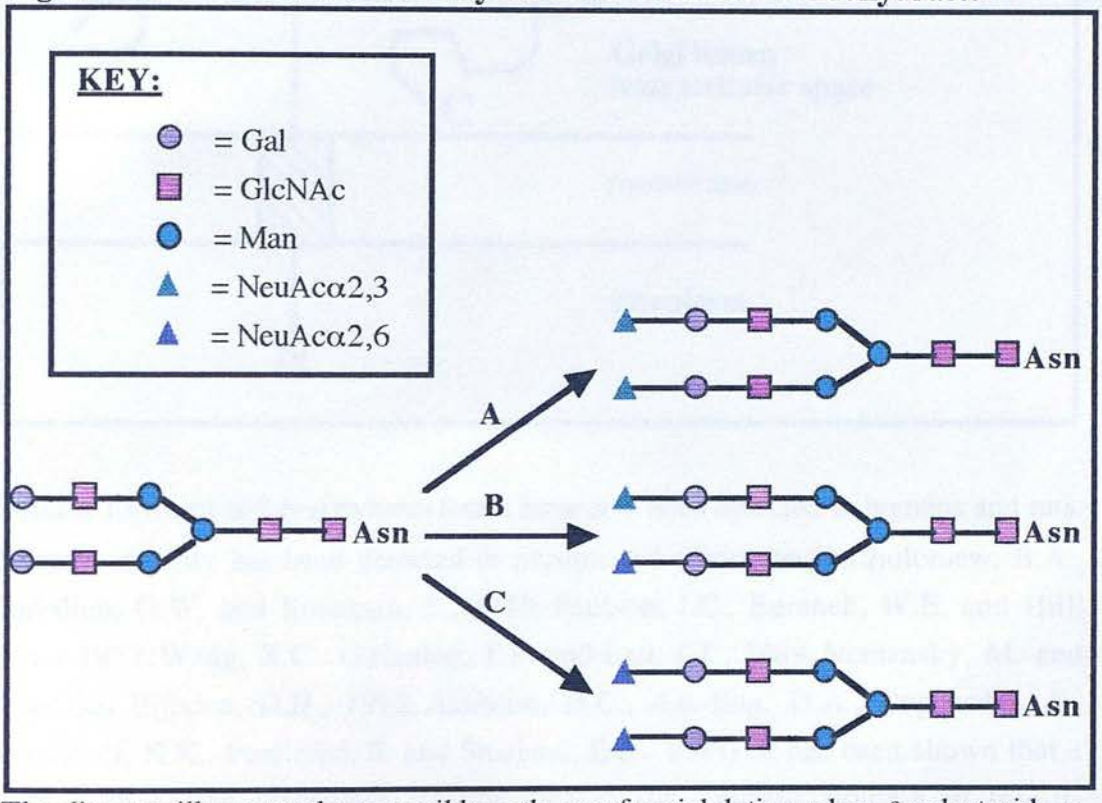
β-galactoside-α-2,6-sialyltransferase was first purified from bovine colostrum in 1977(Paulson, J.C., Beranek, W.E. and Hill, R.L., 1977). It is an enzyme normally found in the Golgi apparatus, which catalyses the transfer of sialic acid from its nucleotide sugar Cytidine Mono Phosphate Neuraminic Acid (CMPNeuAc) to substrates with the non-reducing terminal sequence β1,4-Galactose-N-Acetyl Glucosamine (Galβ1,4GlcNAc) (Paulson, J.C., Rearick, J.I. and Hill, R.L., 1977). Another sialyltransferase enzyme, β-galactoside-α-2,3-sialyltransferase also utilises this terminal sequence for transfer of sialic acid, and competes for the same substrate as α-2,6-sialyltransferase when the two enzymes are co-expressed(Lee, E.U., Roth, J. and Paulson, J.C., 1989).

The activity of the α-2,3-sialyltransferase enzyme must therefore be taken into account in any sialyltransferase assay, and a method of identifying α-2,6-sialylated products must be incorporated into any specific assay. Figure 1.2.1 illustrates three possible sialylation patterns of N-linked carbohydrate when the two enzymes are active.

The primary structure of rat α-2,6-sialyltransferase has been predicted from its amino acid sequence using a Hopp-Woods hydrophilicity plot(Weinstein, J., Lee, E.U., McEntee, K., Lai, P.H. and Paulson, J.C., 1987). This indicates an enzyme with a membrane-spanning region of 17 hydrophobic amino acids located 10 residues from the NH_2-terminus with a large catalytic C-terminal domain which is located

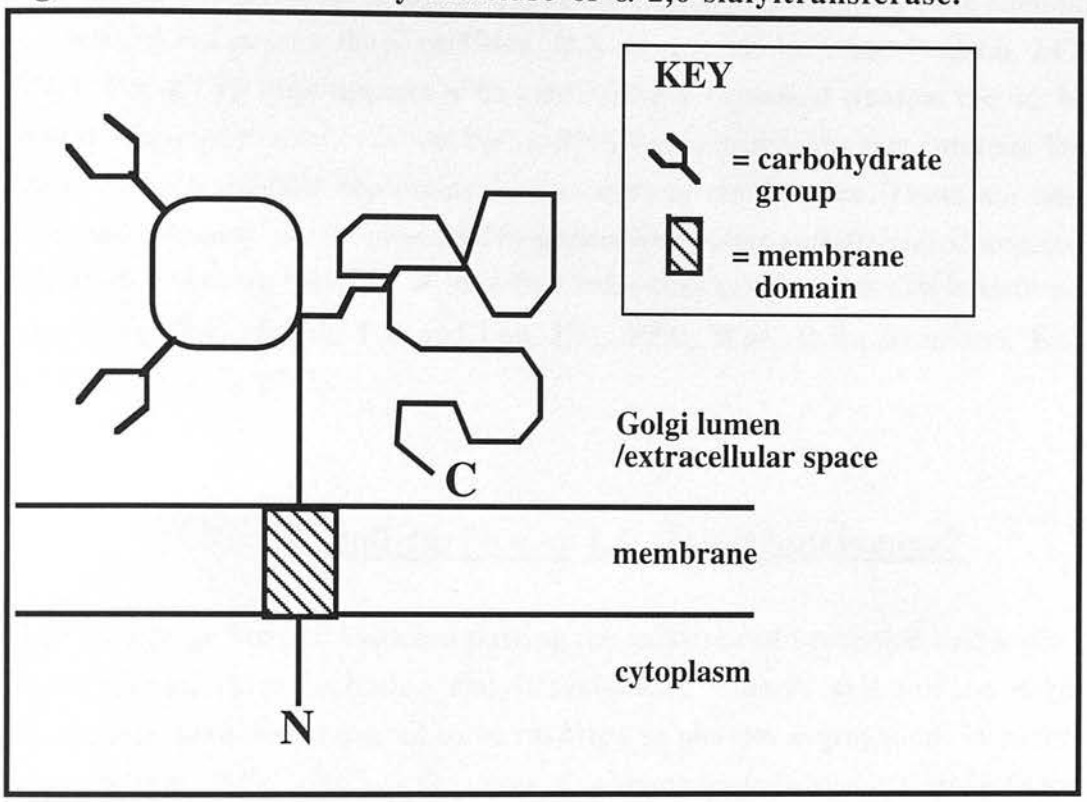
extracellularly or in the lumen of the Golgi Apparatus. This gives an idea of the structure of the human enzyme as the rat enzyme shares 87.6% homology with the human form(Grundmann, U., Nerlich, C., Rein, T. and Zettlmeissl, G., 1990). Like the rat form, the human enzyme contains a short cytoplasmic domain followed by a 15 residue transmembrane domain and a hydrophilic catalytic domain with two potential N-linked glycosylation sites. A schematic view of this primary structure is illustrated in figure 1.2.2.

Figure 1.2.1 Terminal sialylation of N-linked carbohydrate.



The diagram illustrates three possible pathways for sialylation when β-galactoside-α-2,6-sialyltransferase, and β-galactoside-α-2,3-sialyltransferase compete for the same substrate. Pathway A illustrates the action of β-galactoside-α-2,3-sialyltransferase alone with no competition for the substrate, pathway B illustrates a possible outcome when both enzymes are active, and pathway C shows α-2,6-sialylation of the same substrate in the presence of β-galactoside-α-2,6-sialyltransferase alone.
Abbreviations: Gal = galactose, GlcNAc = NAcetylglucosamine, Man = mannose, NeuAc = neuraminic acid (sialic acid).

Figure 1.2.2 Primary structure of α-2,6-sialyltransferase.



Soluble forms of α-2,6-sialyltransferase have also been detected in humans and rats. Enzyme activity has been detected in plasma and colostrum(Bartholomew, B.A., Jourdian, G.W. and Roseman, S., 1973; Paulson, J.C., Beranek, W.E. and Hill, R.L., 1977; Wang, X.C., O'Hanlon, T.P. and Lau, J.T., 1989; Nemansky, M. and Van den Eijnden, D.H., 1992; Aasheim, H.C., Aas-Eng, D.A., Deggerdal, A., Blomhoff, H.K., Funderud, S. and Smeland, E.B., 1993). It has been shown that a Cathepsin D-like lysosomal enzyme cleaves the catalytic domain of α-2,6-sialyltransferase from the transmembrane domain and releases the catalytic domain as a soluble form of the enzyme(Jamieson, J.C., McCaffrey, G. and Harder, P.G., 1993). It has also been shown that levels of soluble α-2,6-sialyltransferase are higher during the acute phase response, and it is thought that increased levels of the cleaving enzyme are induced during the acute phase response(Lammers, G. and Jamieson, J.C., 1989; Harder, G., Jamieson, J.C. and Woloski, B.M., 1990).

Multiple transcripts of α-2,6-sialyltransferase mRNA have also been identified in rat tissues. mRNAs of 3 different sizes - 4.3 kb, 4.7 kb and 3.6 kb have been detected(Paulson, J.C., Weinstein, J. and Schauer, A., 1989). All have high homology and have been shown to be generated by alternate splicing from a single

gene(O'Hanlon, T.P., Lau, K.M., Wang, X.C. and Lau, J.T., 1989). The 4.7 kb message has been shown to be identical to that of the 4.3 kb mRNA with the addition of 2 untranslated exons at the 5' end(Wen, D.X., Svensson, E.C. and Paulson, J.C., 1992). The 4.7 kb form appears to be constitutively expressed whereas the 4.3 kb form is restricted to liver cells, but both mRNA forms encode the same protein. The 3.6 kb mRNA actually represents 3 transcripts of similar size. These are only expressed in kidney, and are generated by alternative splicing and alternative promoter utilisation. There are therefore at least five transcripts of the α-2,6-sialyltransferase gene(Wang, X.C., Smith, T.J. and Lau, J.T., 1990; Wen, D.X., Svensson, E.C. and Paulson, J.C., 1992).

1.3 A cell-surface α-2,6-sialyltransferase?

There is a large body of evidence proving the existence of functional cell-surface glycosyltransferases including sialyltransferases. Indeed, cell-surface sialyltransferases have been reported to be involved in platelet aggregation, in gamete adhesion(Shur, B.D., 1982), in the repair of neuraminidase-treated cell-surfaces and in the specific clearance of desialysed plasma proteins by the liver(Shur, B.D. and Roth, S., 1975). Cell-surface sialyltransferase on erythrocytes is also thought to be a major source of serum sialyltransferase activity(Kim, Y.S., Perdomo, J., Bella, A. and Nordberg, J., 1971). It has been shown that sialyltransferases on the surface of human sperm cells mediate the adhesion of the sperm to the ovum, and that patches of intense sialyltransferase activity on the surface of the ovum correspond to sites of sperm penetration(Daunter, B. and Newlands, J., 1981; Shur, B.D., 1982). Sialyltransferase activity has also been detected on the surface of embryonic chick cells, and it has been shown that neuraminidase treatment of these cells leads to an increased rate of cell re-aggregation. This is thought to be mediated by an increased exposure of non-sialylated sites which act as receptors for cell-surface sialyltransferases, as re-aggregation is inhibited by the addition of glycoproteins possessing the same terminal sugar residues as the neuraminidase-treated cells. There is also evidence that lymphocytes possess numerous cell-surface glycosyltransferases, and that transferases on cytotoxic T-cells may participate in T-cell cytolysis of their target cells. It has been shown that the increase in T-cell surface galactosyltransferase activity is directly proportional to the increase in the ability of these T-cells to bind target cells (Shur,B.D., 1982). Cell-surface galactosyltransferase activity is increased by 5-fold on metastatic murine melanoma cells (Passanati, A. and Hart, G.W., 1990), and

has been found on embryonal carcinoma cells, mesenchymal cells, mouse embryo, melanoma cells, neural crest cells and on mouse sperm cells. It has also been shown to bind to laminin and to promote cell spreading, but not cell adhesion, on laminin-coated surfaces. This enzyme has also been found on the surface epithelium of human stomach cells(Feizi, T., Thorpe, S.J. and Childs, R.A., 1987).

However, despite all the evidence indicating the existence of cell-surface glycosyltransferases, in 1992 it was shown that CDw75 is unlikely to be a cell-surface sialyltransferase. It was discovered that transfection of β-galactoside-α-2,6-sialyltransferase into COS cells not only produces expression of CDw75, but also CD76, and an epitope defined by the mAb HB-6(Bast, B.J., Zhou, L.J., Freeman, G.J., Colley, K.J., Ernst, T.J., Munro, J.M. and Tedder, T.F., 1992; Keppler, O.T., Moldenhauer, G., Oppenlander, M., Schwartz-Albiez, R., Berger, E.G., Funderud, S. and Pawlita, M., 1992; Munro, S., Bast, B.J., Colley, K.J. and Tedder, T.F., 1992). Moreover, it was shown that the sialyltransferase enzyme is not found on the cell surface, but is localised to the perinuclear Golgi region(Bast, B.J., Zhou, L.J., Freeman, G.J., Colley, K.J., Ernst, T.J., Munro, J.M. and Tedder, T.F., 1992). In addition, all three antigens were shown to be sensitive to neuraminidase treatment, and CD76 has been identified as a sialylated glycolipid(Munro, S., Bast, B.J., Colley, K.J. and Tedder, T.F., 1992). All things considered, it seems more likely that CDw75 is not a cell surface sialyltransferase, but is in fact a carbohydrate epitope on a cell-surface glycoprotein or glycoproteins, which is generated by the intracellular activity of β-galactoside-α-2,6-sialyltransferase.

The three sets of mAbs (CDw75, CD76 and HB-6) exhibit similar but not identical binding patterns, indicating that although all epitopes are generated by the same enzyme, they must stain different sialylated glycoproteins and glycolipids on the cell surface whose expression is under another level of control. However, the fact that expression of all three antigens is greatest in mature B-cells, moderate in T-cells and negative in monocytes, must reflect β-galactoside-α-2,6-sialyltransferase activity levels in these cell types(Bast, B.J., Zhou, L.J., Freeman, G.J., Colley, K.J., Ernst, T.J., Munro, J.M. and Tedder, T.F., 1992). It has also been suggested that since the CDw75 antigen is resistant to formalin fixation and paraffin embedding - procedures which very often denature cell-surface proteins - that CDw75 may also be a sialylated glycolipid(Bast, B.J., Zhou, L.J., Freeman, G.J., Colley, K.J., Ernst, T.J., Munro, J.M. and Tedder, T.F., 1992; Munro, S., Bast, B.J., Colley, K.J. and Tedder, T.F., 1992).

1.4 The Importance of Carbohydrate.

One of the major changes associated with cell differentiation and ontogenesis is the altered structure of the carbohydrate chains attached to glycoproteins and glycolipids(Feizi, T., 1985). There is strong evidence that terminal glycosylation sequences are involved in cell motility and in cell-cell communications(Rademacher, T.W., Parekh, R.B. and Dwek, R.A., 1988; Springer, T.A., 1990). In embryonic development, glycoproteins known as cell adhesion molecules (CAMs), influence the specific adhesion of neural cells and liver cells. The glycosylation patterns on these CAMs have been shown to play a major role in the adhesion events mediated by these molecules(Livingston, B.D., De Robertis, E.M. and Paulson, J.C., 1990; Feizi, T., 1991). Sialic acid has been shown to be important in the control of the homophilic binding of neural cell adhesion molecules (N-CAMs), and there are differences between the glycosylation patterns of the embryonic and adult forms, particularly with respect to the extent and nature of sialylation of the N-linked oligosaccharides (Rademacher, T.W., Parekh, R.B. and Dwek, R.A., 1988; Kimber, S.J., 1990). Another CAM, endothelial leucocyte adhesion molecule (ELAM-1) mediates the adhesion of circulating leucocytes to the vascular endothelium. This adhesion has also been shown to be dependent on sialylation of the lactosaminoglycan receptor for ELAM-1(Lowe, J.B., Stoolman, L.M., Nair, R.P., Larsen, R.D., Berhend, T.L. and Marks, R.M., 1990). Differences in carbohydrate structures have also been implicated in many diseases. For example, in patients with diabetes there is a lack of sialic acid in the glomerular basement membrane(Cohen-Forterre, L., Andre, J., Mozere, G., Peyroux, J. and Sternberg, M., 1990), and in patients with arthritis, Crohn's disease and tuberculosis, there are differences in the galactosylation patterns of IgG(Rademacher, T.W., Parekh, R.B. and Dwek, R.A., 1988).

There are many reports on the involvement of carbohydrate on the tumour cell surface. The sugar chains of cell-surface glycoproteins and glycolipids are thought to play an important role in the invasiveness and metastasis of tumour cells, and it is known that tumour cells contain more complex and heavily sialylated carbohydrate on their membrane glycoproteins(Smets, L.A. and Van Beek, W.P., 1984; Yamashita, K., Ohkura, T., Tachibana, Y., Takasaki, S. and Kobata, A., 1984; Saitoh, O., Piller, F., Fox, R.I. and Fukuda, M., 1991). In fact, it has been suggested that sialic acid may be an important factor in the process of metastasis formation(Yogeeswaran, G. and Salk, P.L., 1981; Dennis, J.W., 1986). When glycopeptides from tumour cells are analysed by gel filtration, an overall increase in molecular weight is manifested by

shifts in the elution profiles compared to controls. After treatment with neuraminidase, the differences between tumour glycoproteins and controls disappear in most comparisons(Smets, L.A. and Van Beek, W.P., 1984). In addition, the observed alterations in cell surface carbohydrates occur independent of the histogenetic origin of the cells and the transforming principle involved.

The appearance of increased levels of larger cell-surface carbohydrates is a general accompaniment of tumourigenic transformation, but tumour-specific differences have also been detected in glycolipids and glycoproteins released by the tumour into the circulation, and high serum levels of sialylated proteins and lipids have been detected in cancer patients(Smets, L.A. and Van Beek, W.P., 1984). Increased levels of sialyltransferase activity have also been detected in the PBL of patients with multiple myeloma(Cohen, A.M., Allalouf, D., Bessler, H., Djaldetti, M., Malachi, T. and Levinsky, H., 1989), galactosyltransferase activity levels are increased in ovarian cancer(Uejima, T., Uemura, M., Nozawa, S. and Narimatsu, H., 1992; Uemura, M., Sakaguchi, T., Uejima, T., Nozawa, S. and Narimatsu, H., 1992), and fucosyltransferase expression is altered in certain colon cancers(Stroup, G.B., Anumula, K.R., Kline, T.F. and Caltabiano, M.M., 1990). It has also been shown that the oncogene c-Ha-*ras* induces an almost two-fold increase in α-2,6-sialyltransferase activity in transformed NIH3T3 cells as compared to normal NIH3T3 cells(Vandamme, V., Cazlaris, H., Le Marer, N., Laudet, V., Lagrou, C., Verbert, A. and Delannoy, P., 1992). When injected into mice, these transformed cells produced large tumours at the site of injection and micro metastases in the lungs of the mice.

There is much controversy about the structure of carbohydrate on tumour cells. The causes of the changes are poorly understood, and there are no clear-cut differences that separate a normal cell phenotype from a tumour cell phenotype, and it not known what effect such changes may have on the metastatic potential and aggressiveness of the tumour cell. However, it is possible that the changes in structure observed may be due to the fact that metastatic cells can be of transient phenotypes with properties which are not necessarily the same as those of the cells of the primary tumour, nor by those of the metastatic nodule. In addition, cell-surface carbohydrates are capable of mediating the adhesion of tumour cells to the endothelium of the target organ, and increased sialylation may help in this process. In some animal tumours, cell-surface glycoconjugates have been detected that effectively mask histocompatibility antigens. This may reduce the ability of NK cells to recognise these cancer cells, as increased

sialylation of cell-surface glycoconjugates may confer resistance to NK-cell-mediated lysis of tumour cells(Smets, L.A. and Van Beek, W.P., 1984).

In general, the importance of the role of carbohydrate is often not appreciated. The fact that the addition of sugar chains to glycoproteins and glycolipids can completely alter the size, shape, charge and ability to recognise receptors cannot be ignored. The glycosyltransferases provide an additional level of control over the expression of cell-surface components as a change in the glycosylation pattern of one or more cell-surface proteins or lipids can completely change cell-cell communication networks, growth regulation, host-pathogen interactions, adhesion and motility. It is a challenging area to work in, and hopefully as the importance of carbohydrate becomes more accepted, the complexities of glycosylation patterns and their functions will become better understood.

1.5 Aims of the project.

The initial aim of the project was to determine the identity, function, tissue distribution and regulators of expression of CDw75. As the antigen was originally thought to be a cell-surface β-galactoside-α-2,6-sialyltransferase, and as so little is known about this enzyme in humans, my approach was to attempt to isolate the antigen in order to find its molecular weight and to assay it for sialyltransferase activity. I then expected to be able to look for different isoforms of the enzyme in different human tissues and to determine if different forms had different enzyme activities and different regulators of expression. Isolation of the antigen proved impossible, and I decided to attempt to raise IgG mAbs against human β-galactoside-α-2,6-sialyltransferase in order to immunoprecipitate the antigen.

However, as it became apparent during the course of the project that CDw75 was not a cell-surface sialyltransferase, the aims of the project changed entirely. Effectively, the project became not only a study of CDw75, but also of β-galactoside-α-2,6-sialyltransferase. As such, the aims were to further define the distribution and carbohydrate structure of CDw75, and to compare expression of CDw75 on resting and activated cells and on normal and tumour cells and tissues. At the same time it was aimed to compare CDw75 distribution with that of β-galactoside-α-2,6-sialyltransferase. More specific aims are listed below.

1.5.1 Tissue distribution of CDw75.

Further characterisation of CDw75 expression pattern on lymphocytes using all available CDw75 mAbs. This includes normal lymphocytes, lymphocytes from patients with B-cell lymphocytic leukaemia (B-CLL) and Burkitt lymphoma cell lines representing B-cells at different stages of maturation. It was also aimed to define CDw75 expression on subsets of normal PBL in both resting and activated states.

On solid tissues it was aimed to determine CDw75 expression in both lymphoid and non-lymphoid organs, and to compare expression patterns in normal tissues with that of tumour tissues.

1.5.2 Epitope structures of CDw75 antigen.

Further characterisation of carbohydrate structures of individual epitopes recognised by each mAb using glycosidic enzymes and inhibitors of glycosylation to determine if all epitopes are sialylated, and if carbohydrate is N-linked.

1.5.3 Biochemical analysis of CDw75.

CDw75 has not previously been isolated biochemically. It was aimed to use a variety of detergents to gently solubilise the antigen from CDw75$^+$ cell membranes and to use the available mAbs to immunoprecipitate the antigen from these preparations, and finally determine its molecular weight by electrophoresis.

1.5.4 Manufacture of mAbs specific for human β-galactoside-α-2,6-sialyltransferase.

Using synthetic peptides constructed from the amino acid sequence of human β-galactoside-α-2,6-sialyltransferase, it was aimed to raise mAbs specific for this enzyme in order to isolate and characterise it and to compare its tissue distribution, and cellular distribution with that of CDw75. Specifically it was aimed to raise mAbs of IgG isotype as these are more useful for immunoprecipitation.

1.5.5 Development of an assay for α-2,6-sialyltransferase activity.

Development of a simple assay for β-galactoside-α-2,6-sialyltransferase activity to be used to measure enzyme activity in plasma samples and in immunoprecipitates from the specific mAbs. Several assays exist in which final products of enzyme activity need to be separated using complex chemical methods. It was aimed to use an α-2,6-sialic acid specific lectin to identify specific assay products in a quantitative manner.

1.5.6 Participation in the Fifth International Leucocyte Typing Conference.

It was aimed to use the CDw75 and CD76 panels from the Leucocyte Typing Workshop to compare the tissue distribution and epitope structure of CDw75 and CD76 antigens. It was also aimed to analyse the new mAbs of the B-cell panel in an attempt to identify any new putative CDw75 mAbs. In addition if the mAbs against α-2,6-sialyltransferase were ready in time, it was aimed to submit them for analysis by the workshop.

CHAPTER 2

Materials and Methods.

2.1 Monoclonal Antibodies.

The mAbs listed were used in for immunofluorescent labelling of cells for flow cytometry (Chapters 3 and 7), for immunolabelling of Western blots and immunoprecipitation of labelled antigens (Chapter 5), and for immunohistochemical staining of tissue sections (Chapters 4, 6 and 7).

The CDw75 mAb - HH2, OKB4, EBU-141, and EBU-65 were kindly provided by the originators of the clones(Dorken, B., Moller, P., Pezzutto, A., Schwartz-Albiez, R. and Moldenhauer, G., 1989). The CD45RA mAb (F8-11-13) and the CD14 mAb (VIM13) were obtained from the Third and Fourth Leucocyte Typing Workshops respectively. The CD45RO (UCHL-1), CD3 (UCHT-1) and CD8 (UCHT-4) mAb were generous gifts from Dr.P.Beverley. The mAbs against CD4, CD19, CD29, CD54 and CD58 were purchased from Serotec. L26 (CD20), MT1 (CD43), KP-1 (CD68), EMA (Epithelial Membrane Antigen), CK (cytokerratin) and QBEND (HEV) were purchased from DAKO.

2.2 Expression and Structure of CDw75 on Lymphocytes.

Single-colour and two-colour immunofluorescence staining were used for flow cytometric analysis of cell suspensions in the tissue distribution studies described in chapters 3 and 7. In some experiments, cells were treated with neuraminidase,or cultured in the presence of tunicamycin or P.H.A. prior to staining.

2.2.1 Single-Colour Immunofluorescence Staining.

Cells were harvested from culture and washed twice in PBS 1% BSA. Viability was assessed, and cells were used only if viability was >85%. Cells were dispensed into capped Falcon tubes in aliquots of 2.5 x 10^5 cells before addition of 50 μl aliquots of primary mAb. Tubes were agitated and incubated at room temperature for 45 mins. Cells were then washed twice in PBS 1% BSA before addition of 100 μl of 1/100 $F(ab)_2$ FITC-sheep-anti-mouse IgG (Sigma F2883). Tubes were incubated at room temperature for a further 30 mins and then washed twice more in PBS 1% BSA. The pellets were carefully resuspended before fixing each aliquot in 0.5 ml of PBS + 1% formaldehyde to give a final concentration of 5 x 10^5 cells/ml. Flow cytometric analysis was carried out on a benchtop flow cytometer (FACScan, Becton Dickinson) equipped with an argon-ion laser emitting light at a fixed wavelength of 488nm. Light scatter signals were collected in linear mode, and fluorescence output in logarithmic mode.

2.2.2 Two-Colour Immunofluorescence Staining.

Where two colour immunofluorescence staining was employed, only highly viable cell populations were used (viability >95%). CDw75 mAbs (which are all of IgM isotype) were detected using FITC-anti-mouse IgM (Sigma) previously absorbed with IgM positive human Burkitt lymphoma cells to remove anti-human Ig reactivity, and minimal residual binding of the antibody to human cells was blocked in each test by the addition of 0.5% of normal human serum. IgG mAb were detected using biotinylated anti-mouse IgG (Sigma) and streptavidin-phycoerythrin (Amersham International). 2A4.5 (an IgG anti-phycoerythrin mAb) and VIM13 (an IgM CD14 mAb) were included in all tests as isotype matched negative controls. All fluorochrome-conjugated reagents were titrated and used at optimal concentrations. In tests with OKB4, cells were pretreated with neuraminidase as described above to remove sialic acid and expose this epitope. Flow cytometric analysis was carried out on a benchtop flow cytometer (FACScan, Becton Dickinson) equipped with an argon-ion laser emitting light at a fixed wavelength of 488nm. Light scatter signals were collected in linear mode, and fluorescence output in logarithmic mode. Automatic compensation was set to eliminate leakage between fluorescence channels 1 and 2. Dot plots and data were produced using the 'Lysis' software package.

2.2.3 Neuraminidase Digestion Of Whole Cells.

Cells were routinely harvested, washed and resuspended to 10^7 cells/ml in normal saline (154 mM NaCl). 25 μl of 2U/ml neuraminidase (Type X *Clostridium perfringens* Sigma) was added per 1 ml of cells to give a final concentration of 0.05U/ml. Cells were incubated with neuraminidase for 30 mins at 37°C with occasional shaking. After this time the cells were washed in PBS containing 1% BSA (Sigma) to remove the neuraminidase prior to immunofluorescence staining. The amount of neuraminidase used was initially determined by a titration as described in Chapter 3.

2.2.4 Tunicamycin Inhibition Of N-Linked Glycosylation.

Cells were harvested and washed in fresh medium and resuspended to ~10^6cells/ml in RPMI 10% FCS + 0.05 U/ml neuraminidase Type X (*Clostridium perfringens*). Cells were incubated for 1 hr at 37°C (or overnight) in an atmosphere of 5% CO_2. After incubation, cells were harvested and washed in normal unsupplemented RPMI FCS before resuspending in medium containing varying concentrations of tunicamycin (0 - 10 μg/ml)(Sigma). Cells were cultured in tunicamycin supplemented medium for 18 hrs before harvesting, washing and immunofluorescence labelling with CDw75

antibodies and sialic acid specific lectins Sambucus Nigra Agglutinin (SNA) and Maackia Amurensis Agglutinin (MAA). Stained cells were analysed by flow cytometry.

2.2.5 PHA Stimulation Of Lymphocytes.

Normal peripheral blood lymphocytes (PBL) were obtained from blood donations from laboratory staff and leucocyte concentrates prepared from normal blood donors. PBL were prepared as follows: blood clotting was induced by the addition of a few drops of 1000 units/ml thrombin (ICN ImmunoBiologicals) in 1M $CaCl_2$, and fibrin strands were removed as the blood clotted using applicator sticks. Bloods were then centrifuged at 1000g for 12 minutes over cushions of Histopaque 1077 (Sigma) in capped tubes, and leucocytes recovered from the interface. Adherent cells were removed by culturing the cells in RPMI 1640 + 10% FCS in plastic tissue culture flasks for 1 hr at 37°C. For activation studies, PBL were cultured in RPMI FCS + 100 μg/ml PHA (Wellcome) at 37°C in an atmosphere of 5% CO_2 in air, and harvested at 24 hour intervals over a 3 or 5 day period for immunofluorescence staining.

2.3 Immunohistochemistry.

The immunohistochemical staining technique described below was used to stain the tissue sections described in chapters 4, 6 and 7.

2.3.1 Immunohistochemical Staining Of Tissue Sections.

Paraffin-embedded tissue samples were cut into 3 μm thick sections and mounted on glass slides. Sections were deparaffinised by immersion in xylene for 10 mins. They were re-hydrated by immersion in decreasing concentrations of ethanol in water (Absolute alcohol -> 74% -> 64% -> water). Endogenous peroxidase activity was blocked by removing sections from the absolute alcohol bath and immersing in 1% H_2O_2 (Sigma) in methanol for 10 mins before continuing with the rehydration process. Hydrated sections were washed in running tap water for 5 mins and trypsinised if necessary. (Sections were immersed in 0.1% trypsin, 0.1% $CaCl_2$ in distilled water pH 7.6 - 7.8 at 37°C for 20 mins). Sections were washed twice for 5 mins in TBS (see appendix A) before incubating in 20% normal rabbit serum (NRS)(SAPU) in TBS for 10 mins to reduce non-specific staining. Serum was drained off, and 100 μl aliquots of primary mAb (diluted optimally in 20% NRS) carefully pipetted on to each section and incubated for 30 mins. Slides were washed twice for 5 mins in a bath of TBS, drained and sections incubated in biotinylated

rabbit-anti-mouse-Ig (DAKO) diluted 1/400 in 20% NRS for 30 mins. Slides were washed twice in TBS as before and incubated in avidin-biotin-peroxidase or avidin-biotin-alkaline phosphatase complexes (DAKO) in TBS for 30 mins. Sections were washed twice more as before, and stained using the appropriate enzyme substrate (see appendix A). Stained sections were counterstained by incubation in a bath of 5% Mayers Haematoxylin for 10 mins and washed in slightly alkaline tap water to allow the blue colour to develop. Sections were dehydrated by immersion in increasing concentrations of ethanol in water (reverse of hydration process described) and left in xylene for at least 30 mins before mounting using a synthetic resin (DPX) to fix coverslips. Stained sections were analysed by light microscopy, and photographed using a 35mm camera attached to the microscope. Magnification given on each photographic plate is that of the microscope objective.

2.4 Biochemical Techniques.

The following techniques were used in the experiments described in chapter 5. Cells were solubilised in detergent prior to electrophoretic separation using SDS PAGE. Separated proteins were then transferred to nitrocellulose by Western blotting and then detected using enzyme labelling. Where immunoprecipitations were performed, whole cells were surface labelled using biotin or metabolically labelled using radioactively labelled methionine and cystein prior to solubilisation. Once separated and transferred to nitrocellulose, these labelled proteins were detected using enzyme labelling or autoradiography. The radioimmunoassay was used to test the integrity of cell-surface epitopes after possible damage by detergent treatments. Likewise, dot-blotting was used to test the integrity of detergent solubilised CDw75 epitopes.

2.4.1 NHSS-Biotinylation Of Whole Cells.

Cells were resuspended to 10^8 per ml in PBS (see appendix A). 50 μl per 1 ml of cells of a 44 mg/ml sulpho-N-hydroxysuccinimide ester of biotin (NHSS-biotin) (Pierce) in PBS solution was added, and the suspension incubated on ice.(Cole, S.R., Ashman, L.K. and Ey, P.L., 1987) After 30 mins, ~5 ml PBS was added and the suspension was mixed by inversion. Biotinylated cells were washed three times in PBS prior to solubilisation.

2.4.2 Metabolic Radiolabelling.

Cells were harvested from tissue culture flasks, washed in methionine and cysteine-free culture medium (DMEM, ICN Flow), and resuspended at 10^7 cells/ml in the same

medium. ^{35}S-labelled methionine and cysteine (Amersham) was added (~100 μCi/ml of cells) and cells were cultured at 37°C in an atmosphere of 5% CO_2, 95% humidified air for 3 hrs. (A tray of water was routinely placed in the incubator with the cells in order to absorb any radioactive sulphide gases emitted from the medium. Samples of this water were tested for radioactivity after each labelling session). Cells were harvested and washed prior to solubilisation. (All washings and spent medium were stored for disposal at a later date once radioactivity had decayed to a safe level).

2.4.3 Solubilisation Of Cells.

Cells were washed twice in PBS and counted. After centrifugation to pellet the cells, the supernatant was discarded before gently resuspending the cells in their own pellet volume. Cells were placed on ice and 1 ml of 0.5% detergent in lysis buffer(Baecher, C.M., Infante, A.J., Semcheski, K.L. and Frelinger, J.G., 1988) (see appendix A) containing protease and glycosidase inhibitors (1 x 50 μl vial of each per 1 ml of detergent) was slowly added per 10^7cells. Cells were gently resuspended and incubated on ice for 30 minutes. Cells were centrifuged in capped tubes at 175g for 10 mins at 4°C and 1 ml aliquots of supernatant fluid were carefully removed into Eppendorf tubes and centrifuged at 5000g for 30 mins to remove any residual intracellular vesicles. The supernatant was carefully removed and stored in aliquots at -20°C. Protease and glycosidase inhibitors were stored at -20°C as pre-prepared cocktails at 20 x normal working concentration. The contents of each cocktail, and their normal working concentrations are given in appendix A (Table A1).

2.4.4 Immunoprecipitation.

Protein-A Method.

Bead Arming.

10 mg of Protein A Sepharose (Sigma) was added to 40 μl of PBS and left to swell for 2 hrs, before adding 40 μl of 1% detergent in lysis buffer. 160 μl of rabbit anti-mouse immunoglobulins (DAKO) was then added to the beads, and incubated on a roller at 4°C for 2 hrs. The armed beads were washed 3 times in 0.5% detergent solution and resuspended in 400 μl to give a 10% volume/volume (v/v) armed bead suspension.

Preclearing.

65 μl of 10% v/v armed bead suspension was added to 500 μl aliquots of biotinylated or radiolabelled cell lysate, and incubated on a roller at 4°C for 1 hr. Beads were centrifuged at 6000g for 20s and the supernatants transferred to new tubes containing

fresh 65 μl aliquots of 10% v/v armed bead suspension, and the above preclearance step was repeated twice more. (SDS PAGE analysis of the 3 pellets from these steps showed that all 3 steps were necessary to remove all cross-reacting material from cell lysates). After step 3, supernatants were transferred to fresh tubes for immunoprecipitation.

Immune complex formation.

4 μl aliquots of ascites fluid was added to 250 μl aliquots of precleared biotinylated or radiolabelled lysate and incubated overnight at 4°C on a roller mixer.

Immunoprecipitation.

Complexed lysate was transferred to fresh tubes containing 65 μl of 10% v/v armed beads and incubated on a roller for 1 hr at 4°C. The beads were centrifuged as before, and washed x 1 in 1% detergent, x 2 in 0.1% detergent, and x 2 in detergent-free lysis buffer. Pellets were then either analysed immediately by SDS PAGE or stored at -40°C for later analysis.

Biorad Immunobeads Method.

Preclearing.

50 μl of Biorad immunobeads was added to each 1 ml of biotinylated or radiolabelled lysate and incubated on a rotary mixer at 4°C for 1 hr. These were centrifuged at 500g for 2 mins and supernatant decanted to fresh Eppendorf tubes containing 50 μl of beads. This step was carried out three times. 100 μl of supernatant was aliquoted into fresh Eppendorf tubes for immunoprecipitations and primary mAb added (2 μl neat ascites or 10 μl culture S/N). These were incubated on a rotary mixer overnight at 4°C.

Immunoprecipitation.

30 μl of immunobeads were added to each tube of complexes and incubated at room temperature for 1 hr on a rotary mixer. Beads were pelleted by centrifuging at 500g for 2 mins and the supernatant was removed. The beads were then washed twice in high salt buffer (see appendix A), twice in low salt buffer, and finally in the same buffer with no added NaCl. 40 μl of reducing sample buffer (see appendix A) was added and samples incubated at 100°C on a hot block for 5 mins before loading onto SDS polyacrylamide gels.

Immunoprecipitation - Dynabeads Method.

Bead arming.

M-450 Tosylactivated Dynabeads (Dynal) were used. Beads were washed in sterile distilled water and resuspended to 4 x 10^8 beads/ml. An equal volume of this suspension was added to a 150 μg/ml solution of purified goat-anti-mouse IgM (Sigma) in 0.05M borate buffer, to give an antibody to bead ratio of approximately 5:1. This was incubated at 22°C for 24 hrs with slow end over end rotation. The Dynabeads were collected using a magnetic particle collector (MPC, Dynal), and the supernatant discarded. The beads were washed sequentially in PBS 0.1% BSA: 3 x 10 mins, 1 x 30 mins, and 1 x overnight at 4°C. The beads were collected using an MPC, the supernatant discarded and the beads resuspended in PBS BSA at a concentration of 4 x 10^8 beads/ml. Beads were stored at 4°C as recommended by the manufacturer.

Bead arming with primary mAbs.

1 μl of ascites was added to each 150 μl aliquot of armed Dynabead suspension and incubated overnight on a roller at 4°C. The beads were washed x 4 for 30 mins at 4°C in PBS 0.1% BSA and stored in PBS BSA 0.02% NaN_3 until use.

Immunoprecipitation.

150 μl of primary mAb coated beads (first washed in PBS 0.1% BSA for 30 mins) was added to 250 μl aliquots of biotinylated or radiolabelled cell lysate and incubated on a roller at 4°C for 24 hrs. Beads were recovered using the MPC and washed x 2 for 30 mins in PBS BSA before analysis by SDS PAGE.

2.4.5 Sodium Dodecyl Sulphate Polyacrylamide Gel Electrophoresis (SDS PAGE).

(Method based on (Laemmli, U.K., 1970)). Samples were incubated in reducing sample buffer (see appendix A) at 100°C for 4 mins before loading on to 10% or 12% gels. 15 μl and 20 μl samples were used for 10-well mini-gels, 30 μl samples for 5-well mini gels, and 200-400 μl samples for single sample-well gels. Molecular weight markers (Amersham International Rainbow markers or ^{14}C-labelled markers) were also diluted 1:1 and boiled in sample buffer and loaded on to each gel. The loaded gels were saturated in tank buffer (see appendix A) in a Biorad Miniprotean 2 TM[11] SDS PAGE tank. A voltage of 200V was applied for 45 mins until the solvent front had reached the bottom of the gel. Gels were removed from between the glass plates and immersed in transfer buffer (see appendix A) for 60 mins before Western blotting.

2.4.6 Western Blotting.

Western blotting was carried out using a Pharmacia dry-blotting system. Gels were placed face up on the cathode on a pad of filter paper saturated in transfer buffer (see appendix A). Transfer buffer-soaked nitrocellulose was then smoothed on top, taking care to eliminate any residual air bubbles from between the layers as they can interfere with the passage of current. Another pad of transfer buffer-soaked filter paper was then smoothed on top before sandwiching the layers together by clamping on the anode. A voltage of 100V was applied for 60 mins in order to transfer the SDS PAGE separated proteins to transfer to the nitro-cellulose. Western blots were blocked by incubation in PBS 5% dried milk powder for 2 hrs at room temperature with rocking, or overnight at 4°C before developing.

2.4.7 Enzyme Labelling Of Western Blots.

Unlabelled Proteins.

Blots were washed x 3 for 5 mins in TBS 0.02% Tween 20 before incubating in primary mAbs optimally diluted in PBS 1% BSA for 1 hr at room temperature. Blots were washed as before in TBS 0.05% Tween 20. Washed blots were incubated in horse-radish-peroxidase (HRP) labelled rabbit anti-mouse Ig (Sigma) diluted 1/1000 in TBS Tween for 1 hr at room temperature, and then washed x 3 in TBS Tween before developing.

Biotinylated Proteins.

Blots were washed as described above and then incubated in avidin-biotin-HRP complexes (DAKO - ABC-HRP kit) made up according to the manufacturers instructions and diluted a further 1/5 in ABC buffer (see appendix A). Blots were then washed x 3 as before and then developed using the DAB or ECL method.

2.4.8 Antigen Detection.

Diaminobenzidine (DAB) Method.

Washed blots were bathed in a minimum volume of substrate solution (see appendix A) and colour development was observed within a few minutes. The reaction was stopped by washing in TBS Tween + NaN_3.

Enhanced Chemiluminescence (ECL) Method.

After labelling of specific protein bands with horse radish peroxidase, blots were incubated with 10 ml of mixed E.C.L. reagents (Amersham International) which contain H_2O_2 + luminol (cyclic diacylhydrazide) + a light enhancer, at room

temperature for 1 min. ECL regents were removed, and the blots exposed to photographic film (Hyperfilm - Amersham International) for 1 - 120 seconds depending on light intensity. Films were developed using a Kodak automatic developing machine.

2.4.9 Autoradiography.

After SDS PAGE of radiolabelled samples, gels were dried on an electric gel drier. Dried gels were exposed to Kodak Scientific Imaging Film (X-OMAT AR) in a cassette in the dark room for as long as was necessary in order to see clear bands on the developed film. Films were developed by immersion in Kodak LX24 developer with shaking for 5 mins, washed for 30 seconds in running tap water, and fixed by immersion in Kodak FX-40 liquid fixer for 3 mins until they became transparent. Films were then washed for 5 - 10 mins in running tap water and dried carefully before analysis.

2.4.10 Radioimmunoassay Of Detergent-Treated Cells.

5 x 10^5 formaldehyde-fixed CDw75 positive cells were incubated with 50 μl of primary mAb + 50 μl 0.5% detergent solution for 45 mins at room temperature. Cells were washed x 2 in PBS 0.1% BSA 0.02% NaN_3 and resuspended in the pellet volume. They were incubated in 50 μl aliquots of 1/100 diluted NEM ^{125}I-anti-mouse-IgM (80,000 cpm/well), (containing 25 μl of human serum/ml to block cross-reactivity with human Ig on cell surfaces) for 30 mins at room temperature. Cells were washed twice as before, resuspended to 100 μl in the same buffer and transferred to sealed tubes before counting radioactivity in a gamma counter.

2.4.11 Dot Blotting.

Nitro-cellulose membrane was soaked in TBS and fitted into the dot-blot apparatus (Biorad). It was then re-wetted and vacuum-dried before adding 100 μl aliquots of coating antigen dissolved in TBS. This was allowed to drain through the membrane by gravity for 1 hr before washing and blocking the membrane in PBS 1% BSA 0.05% Tween 20. 100 μl aliquots of primary mAbs optimally diluted in PBS 1% BSA were added, and allowed to drain through by gravity for 30 mins. Blots were washed overnight in TBS 0.05% Tween 20 at 4°C. Washed blots were incubated in peroxidase-labelled rabbit anti-mouse Ig (Sigma) diluted 1/1000 in TBS Tween for 1 hr at room temperature. They were then washed x 3 in TBS Tween before addition of DAB substrate solution (see appendix A). After colour development, the reaction was stopped by washing in TBS Tween + 0.02% NaN_3.

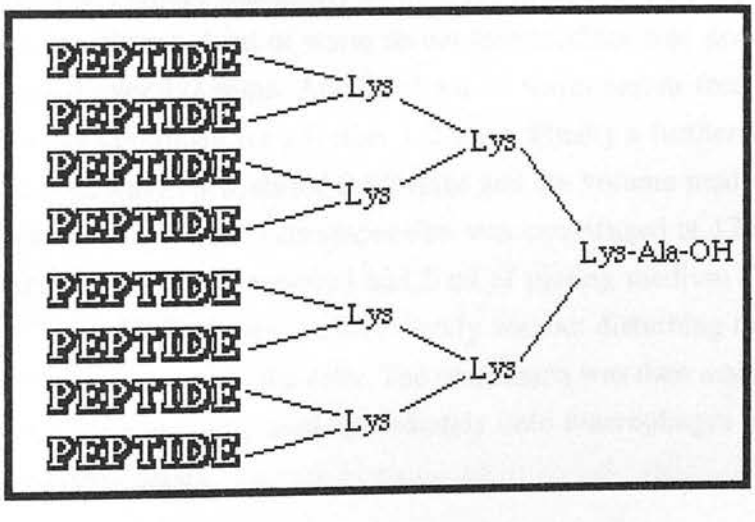
2.5 Monoclonal antibody production.

The following techniques were employed in the production of the Pst mAbs as described in Chapter 6. Synthetic peptides were constructed from the amino acid sequence of human α-2,6-sialyltransferase and used as immunogens for the production of α-2,6-sialyltransferase specific mAbs. mAbs produced were screened using the anti-peptide ELISAs and the Raji-cell-bound ELISA.

2.5.1 Synthetic Peptide Production.

A computer structure prediction program written by E.E.Eliopoulos, (Secondary Structure Prediction Suite Version 2.2, March 1986), was used to analyse the secondary structure of α-2,6-sialyltransferase. The resulting profiles were used to determine which regions of the protein were most likely to a) protrude from the surface of the molecule and b) not be adjacent to glycosylation sites. Three regions were found to fit these criteria, and the amino acid sequences of these regions were used in the construction of 3 Multiple Antigenic Peptides (MAPs) by Peptide and Protein Research at the University of Exeter. Each MAP contains 8 copies of the peptide linked by a poly-lysine core (Illustrated in Figure 2.1). The advantage of using this system is that the complexes are large enough to be used as immunogens without the need for a carrier protein, thus eliminating unnecessary immune reactions to any carrier protein.

Figure 2.1 Diagrammatic Representation Of A MAP.



2.5.2 Antibody Production From Synthetic Peptides.

Immunizations.

Female BALB/C mice were initially immunised intraperitoneally with 50 μg of peptide JP3 in 100 μl 50% Inject Alum (Pierce) in normal saline. 4 booster injections of 100 μl of 500 μg/ml JP3 in saline were given over a period of eight weeks. Serum samples were tested for anti-peptide activity by peptide ELISA, and mice were reboosted twice more at 2 week intervals with 100 μg JP3 in 200 μl of 50% Alum in normal saline. Sera were retested, a final boost of 100 μl of 500 μg/ml peptide in saline given, and the most highly responsive mouse sacrificed 2 days later for fusion.

Fusions.

Macrophages were collected, washed twice, and the suspension made up to 50 ml with HAT medium. Cells were plated out onto 5 x 96-well tissue culture plates (Costar) at a density of 10^3/well and cultured at 37°C in an atmosphere of 5% CO2. . Splenocytes from the sacrificed mouse (typically ~ 10^8 cells/spleen), Sp_20 (a HAT-sensitive mouse myeloma cell-line), and fresh murine macrophages were washed twice in serum-free RPMI. The splenocytes were resuspended in 5 ml of ice-cold 0.17M NH_4Cl for 2 mins in order to lyse any red blood cells present in the sample. This was topped up with serum-free medium and cells were washed once more to remove the lysed cells. Cells were mixed in a ratio of 10 spleen cells : 1 Sp_20 cell and centrifuged at 175g for 5 mins. The supernatant was carefully removed and the pellet tapped loose from the side of the tube. 0.5 ml of 54% polyethylene glycol (PEG-1500 - Koch-Light) in PBS (previously sterilised by autoclaving) was slowly overlayered onto the pellet. The mixture was gently swirled to resuspend the pellet and mix the cells. After 2 mins stirring, 1 ml of warm serum-free medium was slowly added and stirring continued over 1-2 mins. Another 1 ml of warm serum-free medium was added, and stirring continued for a further 1-2 mins. Finally a further 1 ml of warm serum-free medium was added, stirred for 2 mins and the volume made up to 10 mls with warm serum-free medium. This suspension was centrifuged at 175g for 5 mins, the supernatant was carefully removed and 5 ml of plating medium (DMEM (ICN Flow) + 10% FCS + HAT (Sigma)) added slowly without disturbing the pellet. This was swirled gently to resuspend the cells. The suspension was then made up to 50 ml with warm HAT medium and plated immediately onto macrophages in 5 x 96-well tissue culture plates (Costar).

Screening and Cloning.

Plates were fed every 3 days and examined daily 7 days after fusion for the presence of colonies of cells. Supernatants from "positive" wells were carefully removed and tested for the presence of antibody by peptide-ELISA or cell-bound ELISA (using Raji cells). Wells were topped up with fresh DMEM-HAT. Large colonies of antibody secreting cells were transferred to larger wells in 24-well plates to allow further growth. Supernatants were re-screened, and positive colonies were cloned by dilution of cells to 10 cells/ml and seeded into 96 well plates in 100 μl aliquots (1 cell/well). Plates were checked daily for cell growth, and colony supernatants were rescreened by ELISA. Positive clones were expanded by transferring to larger wells and then sequentially larger tissue culture flasks, freezing down samples in liquid nitrogen at each stage to prevent loss of clones in the event of infection.

2.5.3 Peptide ELISAs.

Alkaline phosphatase method.

96 well ELISA plates were coated overnight at 4°C with 100 μl/well of 50 μg/ml of peptide dissolved in Carbonate-Bicarbonate coating buffer pH 9.6 (capsules - Sigma). Plates were washed x 3 in PBS 0.05% Tween 20, and blocked by incubation in PBS 1% BSA for 30 mins at room temperature. Antibody samples were loaded into wells in triplicate in 100 μl aliquots, and incubated at room temperature for 90 mins. Normal mouse serum or irrelevant hybridoma supernatant were used as negative controls. Plates were washed x 3 in PBS Tween before addition of 100 μl aliquots of 1/100 diluted alkaline phosphatase labelled anti-mouse-IgG (Sigma) in PBS 1% BSA. Plates were incubated at room temperature for 90 minutes, and washed x 3 as before. A 200 μl aliquot of substrate solution (see appendix A) was then added to each well and plates were read at 405 nm in a Dynatech MR5000 ELISA plate reader after 30-45 mins.

Horse radish peroxidase (HRP) method.

Plates were coated, blocked and incubated in primary antibody as described above. After washing plates, a 100 μl aliquot of HRP-labelled rabbit-anti-mouse-Ig (DAKO) diluted 1/2000 in PBS 1% BSA was added to each well, and incubated for 90 mins at room temperature. Plates were washed x 3 as before and a 100 μl aliquot of substrate solution (see appendix A) added to each well. When a colour change from clear to yellow was observed (typically within 1-5 minutes) the reaction was stopped by the addition of 100 μl of 4M H_2SO_4/well. Plates were then read on a Dynatech MR5000 ELISA plate reader using a 490 nm test filter and a 630 nm reference filter.

2.5.4 Cell-Bound ELISA.

Adherent cells (e.g. RAJI-A) were grown to confluence, and removed from the culture flask by vigorous pipette action. Cells were counted and made up to 10^6 cells/ml in fresh medium (DMEM, 10% FCS, P/S, Glu). 10 μl of Phorbol Myristate Acetate was added to 50 ml of cell suspension and 100 μl aliquots of cells were seeded into five 96 well flat-bottomed microtitre plates. Cells were grown overnight until they were close to confluence. The plates were then washed twice in PBS pH 7.2 and fixed with 100 μl aliquots of 1% paraformaldehyde in PBS for 30 mins (or until use after storing at 4°C). After fixing or storage, the plates were washed once with TBS 1% BSA and 50-100 μl of mAb was added to wells. Plates were incubated overnight at 4°C and washed twice in TBS 1% BSA before adding 100 µl of biotin-conjugated anti-mouse Ig (1/400 in TBS 1% BSA) per well. After 1 hr at 20°C, plates were washed twice in TBS BSA and two drops of avidin-biotin-HRP complexes (DAKO ABC-HRP kit) in ABC buffer (see appendix A) were added to each well. Plates were incubated at room temperature for 30 mins, and washed twice in TBS BSA before addition of ~200 μl/well of filtered DAB substrate solution (see appendix A). When staining of the cells was evident, they were washed in TBS and examined under an inverted microscope.

2.6 Development of an Assay For α-2,6-Sialyltransferase.

The following assay was developed to specifically measure α-2,6-sialyltransferase activity in Pst immunoprecipitates and in serum samples. Unfortunately, attempts at immunoprecipitation of enzymatically active α-2,6-sialyltransferase were not successful, and are therefore not described in the thesis.

2.6.1 Assay For α-2,6-Sialyltransferase.

This method is adapted from that of Mattox *et al* (Mattox, S., Walrath, K., Ceiler, D., Smith, D.F. and Cummings, R.D., 1992). 96 well ELISA plates were coated overnight at 4°C with 100 μl/well of 1 μg/ml asialofetuin (Sigma) in coating buffer. Plates were then washed x 3 in PBS 0.05% Tween 20 (Sigma) and blocked in PBS 0.05% Tween 20 1% Gelatin (Sigma) for 30 mins at room temperature. Plates were washed x 3 in PBS Tween before setting up the assay. The assay mix in each well consisted of 50 μl of enzyme + 50 μl of 100 μM CMP-NANA (Boehringer Mannheim or Sigma) in 50 mM cacodylate buffer pH 6.5. Standard samples of purified rat α-2,6-sialyltransferase (Sigma) were used in order to obtain a standard curve. Negative controls consisted of wells containing sialic acid + no enzyme, or buffer with no

enzyme or sialic acid present. The mixture was incubated at room temperature for 1 hr. Plates were then washed x 3 in PBS Tween before adding 100 μl aliquots of 1 μg/ml biotinylated Sambucus Nigra Agglutinin (SNA) in PBS Tween. Plates were incubated at room temperature for 1 hr and then washed x 3 as before. 3 drops of avidin-biotin-peroxidase complexes (DAKO) in TBS were then added to each well, and incubated at room temperature for 30 mins. Plates were then washed a further x 3 before addition of 100 μl aliquots of substrate solution (see appendix A). When a colour change from clear to yellow was observed (typically within 1 minute) the reaction was stopped by the addition of 100 μl aliquots of 4M H_2SO_4. Plates were then read on a Dynatech MR5000 ELISA plate reader using a 490 nm test filter and a 630 nm reference filter. A standard curve was obtained by plotting the log of sialyltransferase concentration against the optical density (O.D.) detected by the reader after subtracting the O.D. values of negative controls. Unknown enzyme concentrations were then estimated from the curve. This assay was tested for specificity for α-2,6-sialyltransferase by substitution of SNA with Maackia Amurensis Agglutinin (MAA) - a lectin specific for α-2,3-linked sialic acid residues. O.D. values obtained were lower than those of the negative controls using SNA.

CHAPTER 3

Expression and Structure of CDw75 on Lymphocytes.

3.1 Distribution of CDw75 on Lymphocytes.

As described in chapter 1, all CDw75 mAb have previously been shown to react specifically with mature B lymphocytes which are sIg^+, as well as sIg^+ B-cell lines, leukaemias and lymphomas. Also, mAbs have not been found to react to pre-B-cell lines or to sIg^- B-lineage lymphoblastic leukaemias or sIg^- B-cell lines(Mittler, R.S., Talle, M.A., Carpenter, K., Rao, P.E. and Goldstein, G., 1983; Epstein, A.L., Marder, R.J., Winter, J.N. and Fox, R.I., 1984; Smeland, E., Funderud, S., Ruud, E., Blomhoff, H.K. and T, G., 1985; Dorken, B., Moller, P., Pezzutto, A., Schwartz-Albiez, R. and Moldenhauer, G., 1989; Erikstein, B.K., Beiske, K., Smeland, E.B., Davies, C.D.L., Blomhoff, H.K. and S, F., 1989; Gramatzki, M., Burger, R., Kraus, J., Lauer, U., Rohwer, P., Eger, G., Kalden, J.R. and Henschke, F., 1991). Interestingly CDw75 is not expressed on $sIgM^+$ B-cells from foetal samples which implies that CDw75 is expressed at a later stage of B-cell development than sIg, and as CDw75 is not expressed on EBV transformed lymphoblastoid cell lines or on plasma cell tumours, it has been suggested that CDw75 is lost from the cell surface at a late stage of terminal B-cell differentiation(Dorken, B., Moller, P., Pezzutto, A., Schwartz-Albiez, R. and Moldenhauer, G., 1989). There are also some reports of mAbs HH2, EBU-141, EBU-65 and LN-1 binding to normal peripheral blood T-cells, but no reactivity with T-cell lines or T-cell leukaemias or lymphomas has previously been described(Dorken, B., Moller, P., Pezzutto, A., Schwartz-Albiez, R. and Moldenhauer, G., 1989; Gramatzki, M., Lauer, U., Burger, R., Huber, C., Rohwer, P., Kalden, J.R. and Henschke, F., 1989; Gramatzki, M., Burger, R., Kraus, J., Lauer, U., Rohwer, P., Eger, G., Kalden, J.R. and Henschke, F., 1991).

In order to further characterise CDw75 expression patterns already observed, in sections 3.1.1 and 3.1.2 respectively, I have looked at CDw75 expression on PBL from a number of normal healthy donors and from patients with B-CLL. In section 3.1.3, sublines of the Namalwa Burkitt lymphoma cell-line were also stained with CDw75. These sublines are thought to resemble B-cells arrested at different stages of differentiation(Guy, K., Ross, J.A. and Steel, C.M., 1989; Guy, K., Middleton, P.G., Bansal, N.S., Ross, J.A. and Steel, C.M., 1990), and thus may be of use in further determination of the stage of B-cell differentiation at which CDw75 expression occurs. In addition, as the epitope recognised by the LN-1 mAb is reported to be highly susceptible to neuraminidase treatment(Epstein, A.L., Marder, R.J., Winter, J.N. and Fox, R.I., 1984), I have also looked at the effects of neuraminidase

treatment of cells on mAb binding in most of the samples tested. Neuraminidase catalyses the removal of sialic acid groups from sialylated glycosides of glycoproteins and glycolipids. By pre-treating cells with neuraminidase prior to staining with mAbs, we can assess which, if any of the CDw75 epitopes contain sialylated carbohydrate.

3.1.1 CDw75 Expression on Normal Lymphocytes.

Normal peripheral blood lymphocyte (PBL) samples were obtained from laboratory staff and from leucocyte concentrates (buffy coats) from normal blood donors at the Blood Transfusion Service. Cells were stained by indirect immunofluorescence using 3 mAbs from the CDw75 panel from the 4th International Leucocyte Typing Workshop (Dorken, B., Moller, P., Pezzutto, A., Schwartz-Albiez, R. and Moldenhauer, G., 1989). HH2, OKB4 and EBU-141 are mAbs which were assigned to this cluster. EBU-65 was also used. Although not formally included in the CDw75 cluster, it was in the same preliminary statistical cluster as the others, and does exhibit similar staining characteristics (Gramatzki, M., Lauer, U., Burger, R., Huber, C., Rohwer, P., Kalden, J.R. and Henschke, F., 1989; Guy, K., Ross, J.A. and Steel, C.M., 1989; Johnson, G.D., MacLennan, I.C.M., Khan, M., Hardie, D.L. and Richardson, P.R., 1989). Unfortunately, the fourth mAb of the cluster (LN-1) was not available for us to use.

10 PBL samples were stained using HH2, EBU-141, EBU-65 and OKB4 as well as CD19, CD43, CD4, CD8, CD45RA and CD45RO. Cells were also treated with 0.05 U/ml neuraminidase to remove sialic acid prior to staining. The amount of neuraminidase used was determined by a titration. The results of this titration are illustrated in figure 3.1.1. CDw75 staining patterns were very similar for all normal donors tested, and flow cytometry histograms for cells from three donors are shown in figure 3.1.2. A small percentage of cells (4-7%) correlating roughly with the percentage of B-cells in each sample, were stained with high intensity using HH2 and EBU-141. A small proportion of cells in these samples was also weakly stained with these mAbs. This may be a small subpopulation of T-cells. This subpopulation was more apparent in EBU-65 stained samples. As with HH2 and EBU-141, EBU-65 stained a small number of cells with high intensity. However, a much larger population of cells was found to be weakly stained with this mAb. Binding of all three mAbs was completely abrogated by neuraminidase treatment of cells. OKB4 staining was negligible in all samples tested, and was only slightly increased producing dimly stained cells, after neuraminidase treatment.

Figure 3.1.1 Results of Neuraminidase Titration

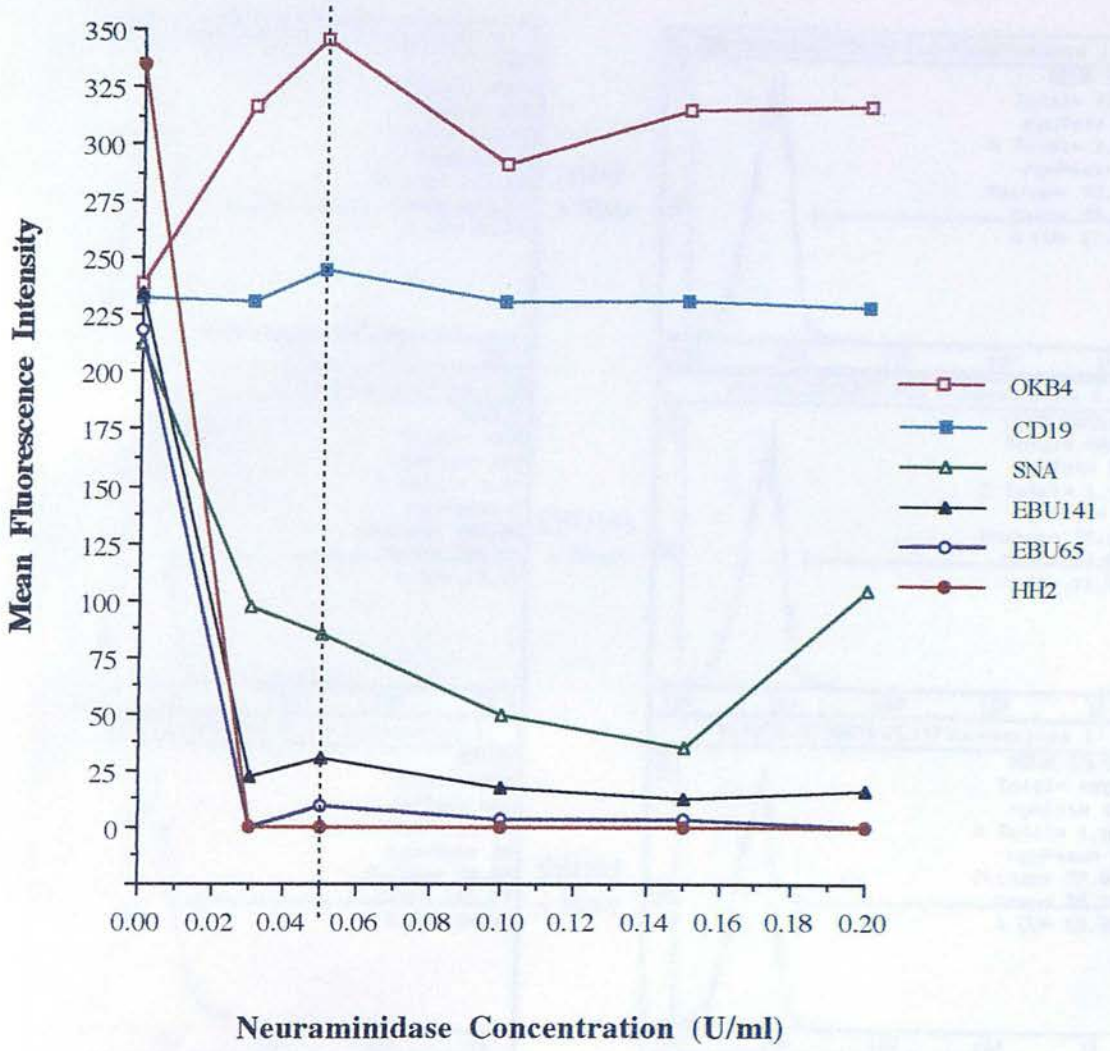


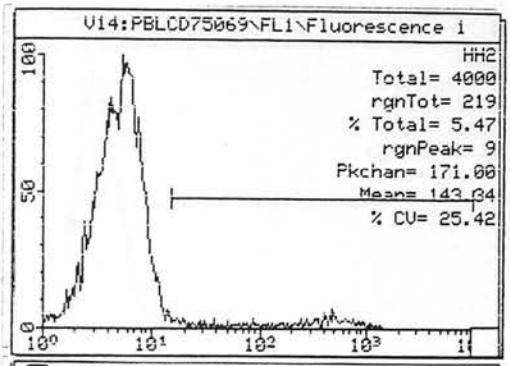
Figure 3.1.1 PNT cells were treated with different concentrations of Neuraminidase Type X (*Clostridium perfringens*) in the range 0 - 0.2 U/ml prior to immunofluorescence staining, and were analysed by flow cytometry. Mean fluorescence intensities of stained cells indicated that 0.05 U/ml was the lowest concentration of Neuraminidase that should be used.

Total numbers of positively stained cells was found to vary a great deal from donor to donor. However, the general pattern of staining was always the same, with EBU-65 staining highest numbers of cells with low intensity, and similar numbers of cells as HH2 and EBU-141 with high intensity. These subpopulations of lymphocytes were examined in more detail later on in the project and are described in section 3.3.

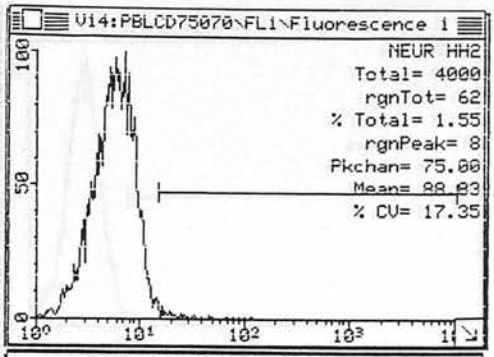
3.1.2(a) CDw75 Expression on Normal Lymphocytes.

Donor 1.

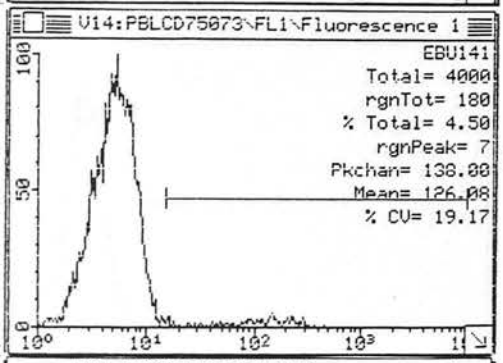
HH2



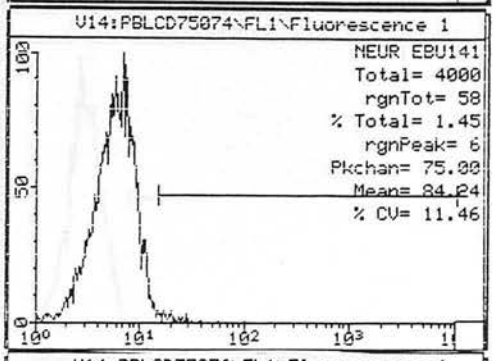
HH2 + Neur



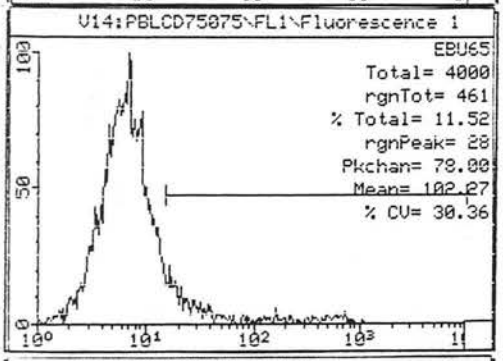
EBU141



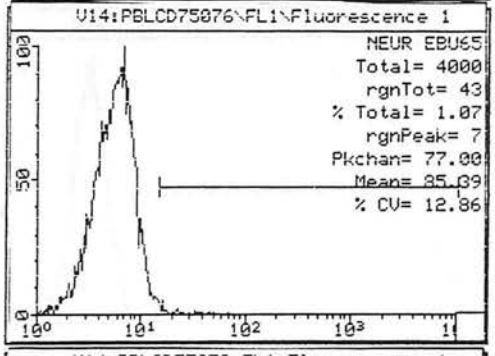
EBU141 + Neur



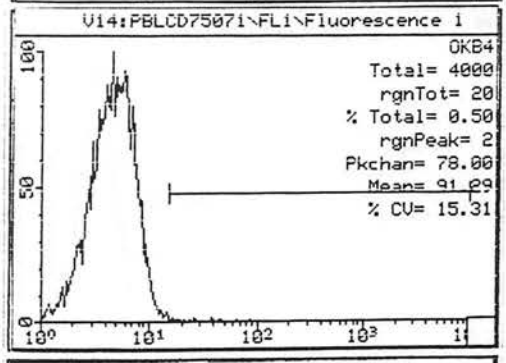
EBU65



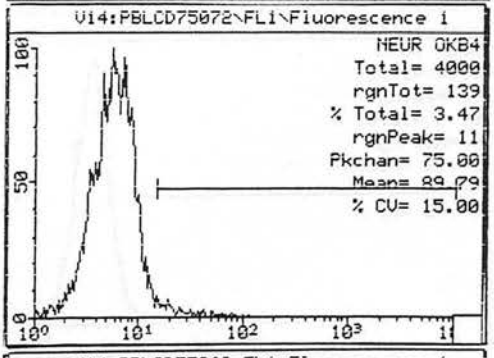
EBU65 + Neur



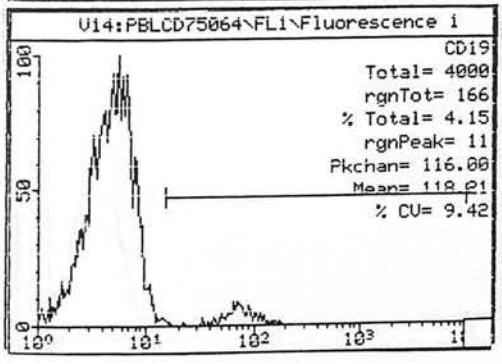
OKB4



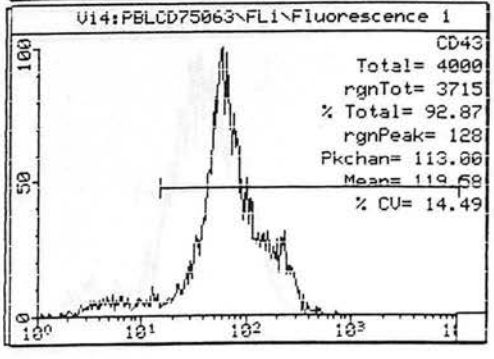
OKB4 + Neur



CD19



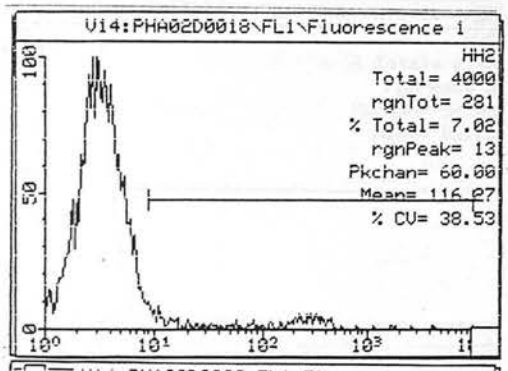
CD43



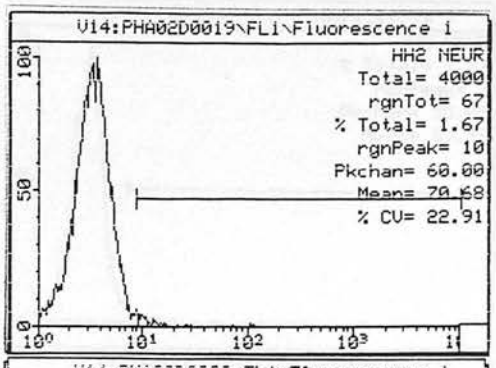
3.1.2(b) CDw75 Expression on Normal Lymphocytes.

Donor 2.

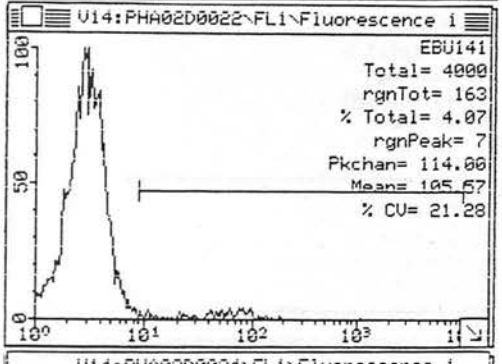
HH2



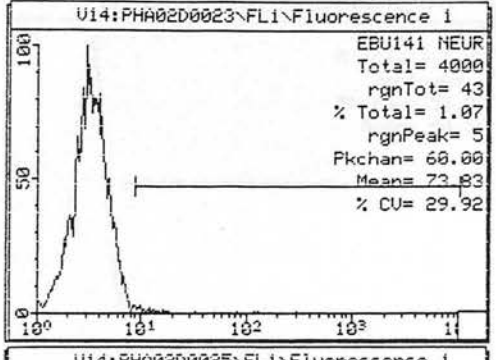
HH2 + Neur



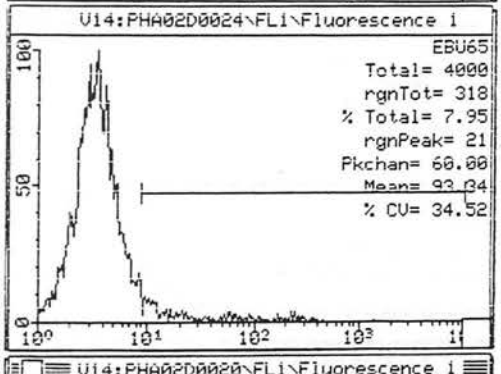
EBU141



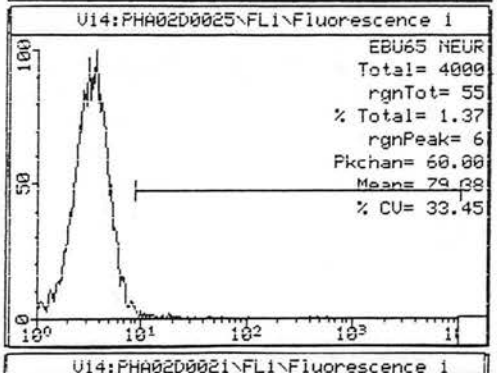
EBU141 + Neur



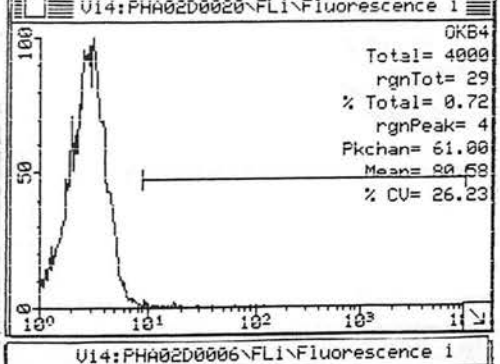
EBU65



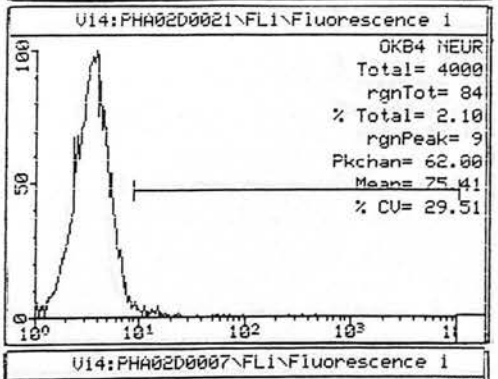
EBU65 + Neur



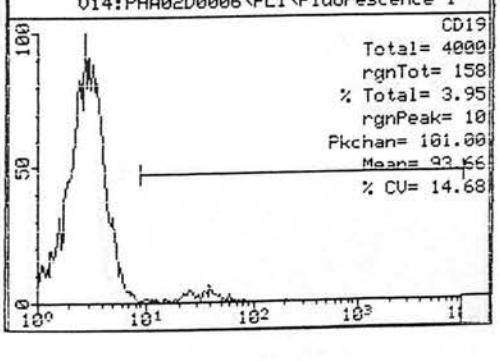
OKB4



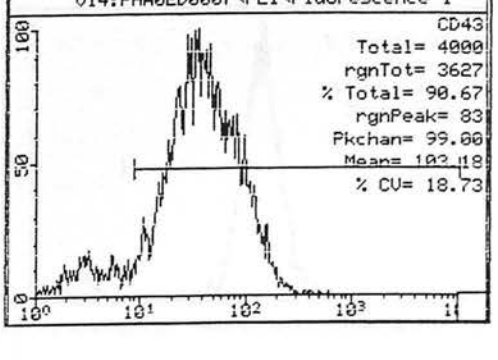
OKB4 + Neur



CD19



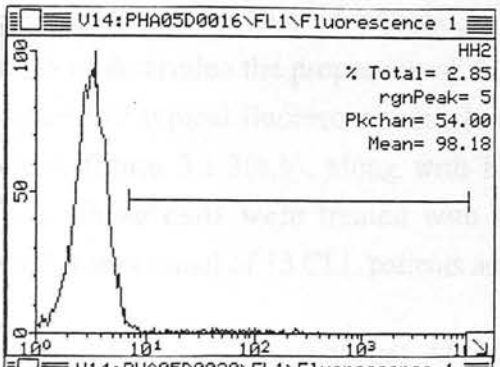
CD43



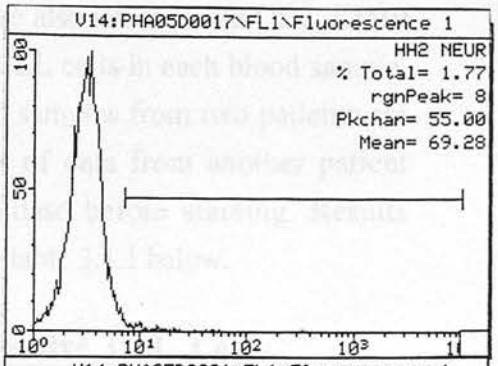
3.1.2(c) CDw75 Expression on Normal Lymphocytes.

Donor 3.

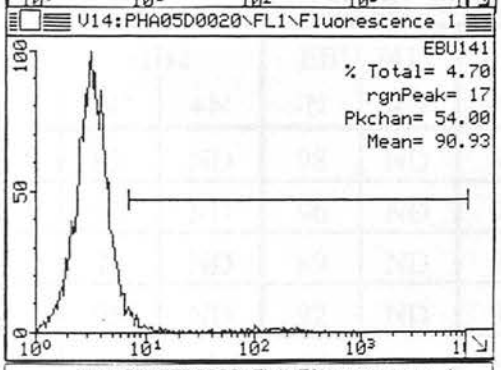
HH2



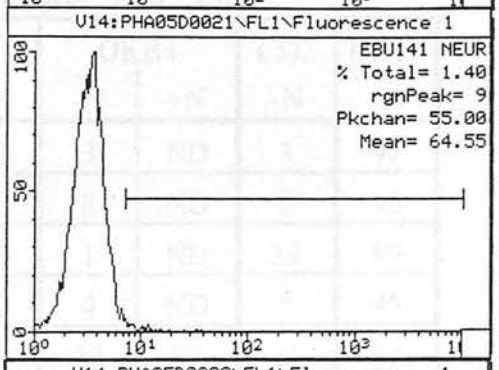
HH2 + Neur



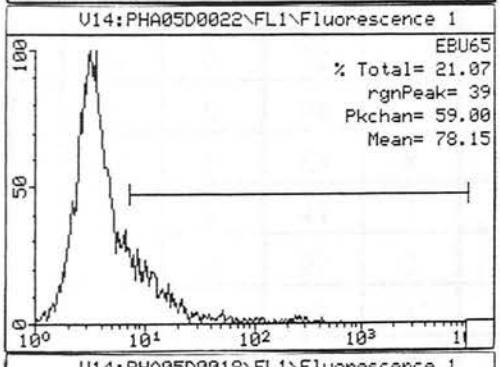
EBU141



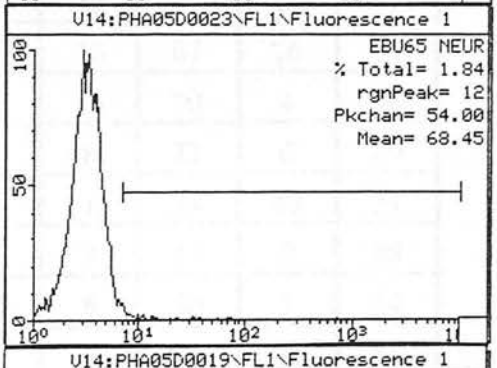
EBU141 + Neur



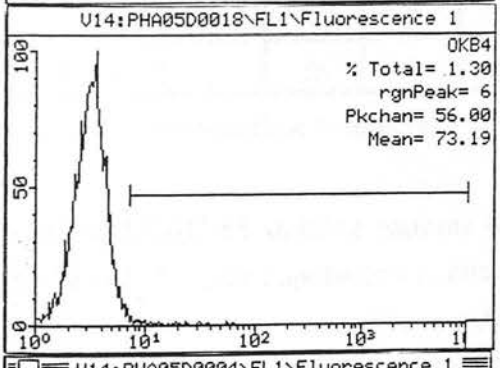
EBU65



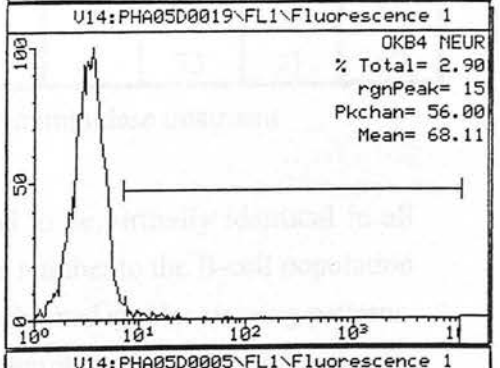
EBU65 + Neur



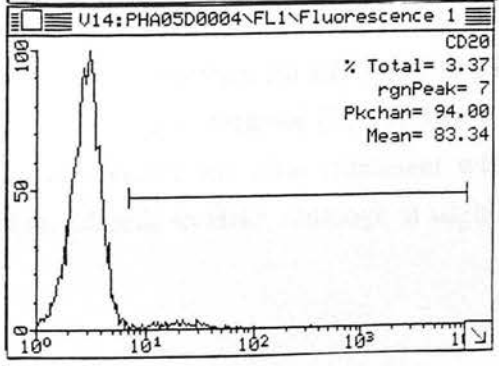
OKB4



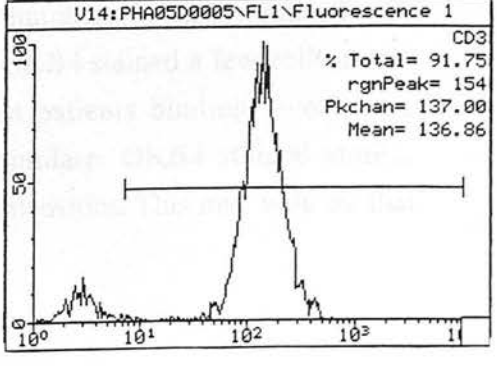
OKB4 + Neur



CD20



CD3



3.1.2 CDw75 Expression in CLL Patients.

Blood samples from 23 patients with B-cell Chronic Lymphocytic Leukaemia (B-CLL) were studied for CDw75 expression. Cells were also stained with CD2, CD19 and CD5 to determine the proportion of T-, B- and CLL cells in each blood sample. Histograms of typical fluorescence levels of stained samples from two patients are shown in figure 3.1.3(a,b), along with histograms of data from another patient 3.1.3(c) whose cells were treated with neuraminidase before staining. Results obtained from a panel of 13 CLL patients are given in table 3.1.1 below.

Table 3.1.1 Percentages of CDw75 Positive CLL Cells.

Patient	HH2		EBU-141		EBU-65		OKB4		CD2	CD19
No.	-N*	+N	-N	+N	-N	+N	-N	+N	-N	-N
1	92	ND	98	ND	96	ND	3	ND	3	97
2	97	ND	96	ND	94	ND	8	ND	6	95
4	78	ND	89	ND	90	ND	1	ND	32	89
5	95	ND	92	ND	92	ND	4	ND	5	95
9	87	1	75	1	88	1	33	77	11	67
11	81	2	72	3	82	2	38	67	16	74
16	92	0	94	1	91	0	16	70	4	94
17	87	1	83	8	84	6	8	72	6	89
18	44	1	44	1	40	1	1	24	40	51
19	77	0	97	2	92	1	3	33	0	98
20	48	0	48	1	36	3	6	50	3	44
21	56	1	63	0	62	0	2	42	26	55
22	58	1	56	1	61	2	6	73	21	60

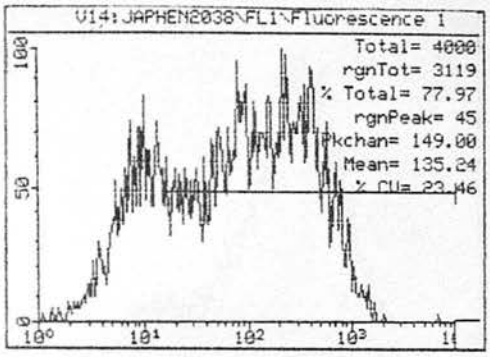
* -N = before neuraminidase treatment; + N = after neuraminidase treatment.

EBU-141 and EBU-65 staining patterns were found to be virtually identical in all samples tested. A major population of cells, similar in number to the B-cell population were brightly stained with both of these mAb. HH2 exhibited similar staining patterns, but in some cases (e.g. Patient 4 in figure 3.1.3) the number of brightly stained cells was slightly lower than for EBU-141 and EBU-65. OKB4 stained a few cells at low intensity in some patients (9, 11, 16), but in most patients binding levels were negligible. However, after treatment with neuraminidase, OKB4 stained similar numbers of cells to HH2, although at slightly lower intensities. This may indicate that

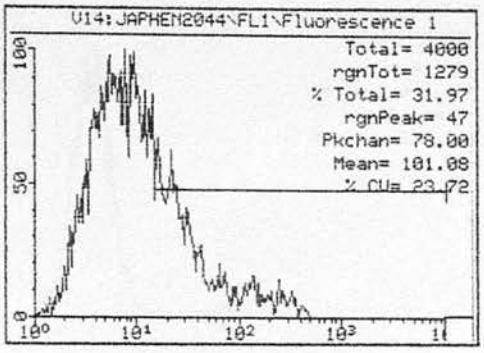
3.1.3(a) CDw75 Expression in CLL Patients.

Patient 4.

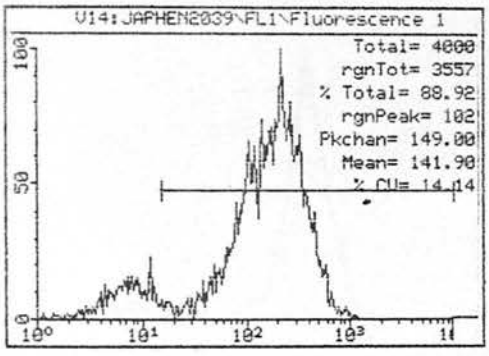
HH2



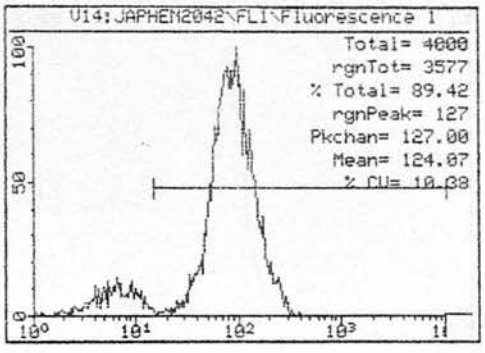
CD2



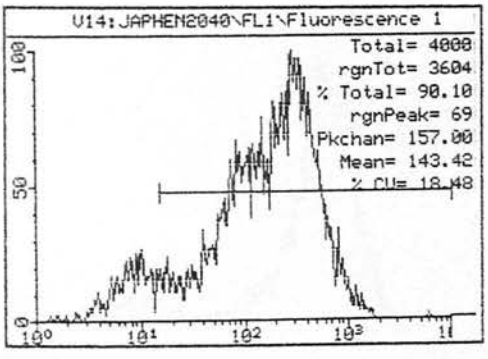
EBU141



CD19



EBU65



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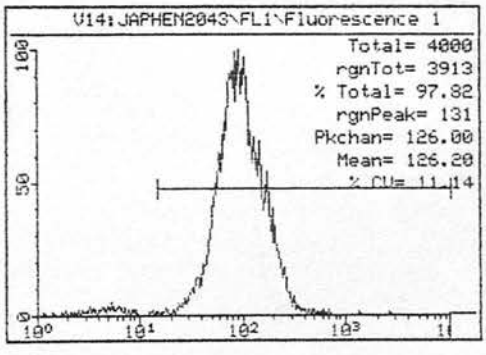
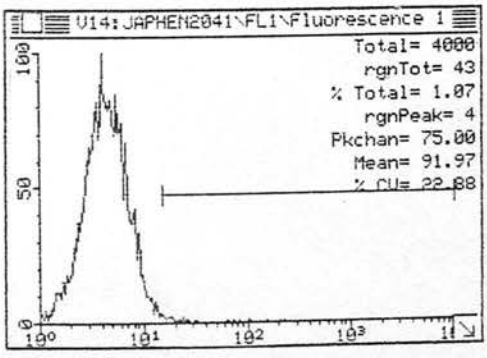


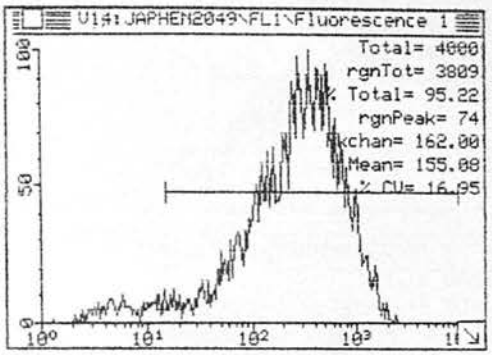
OKB4



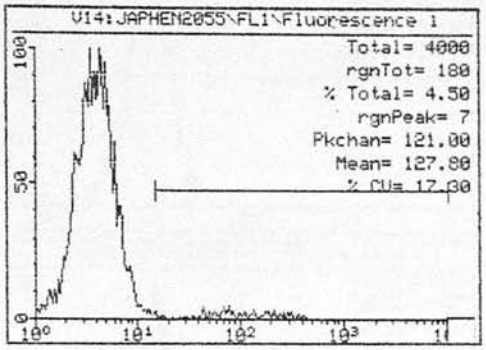
3.1.3(b) CDw75 Expression in CLL Patients.

Patient 5.

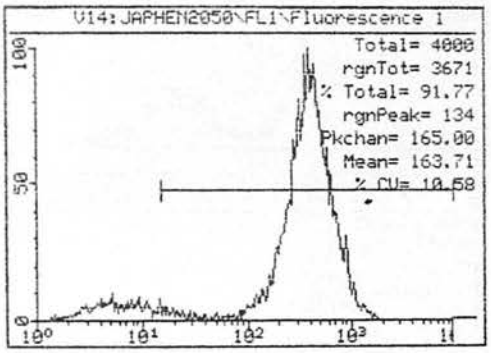
HH2



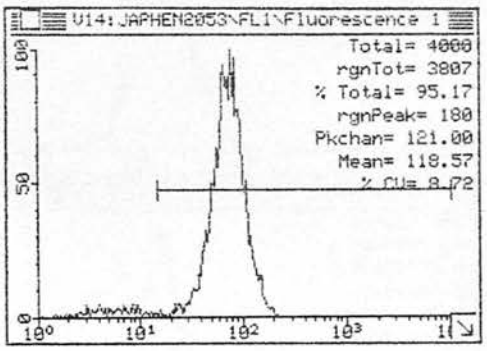
CD2



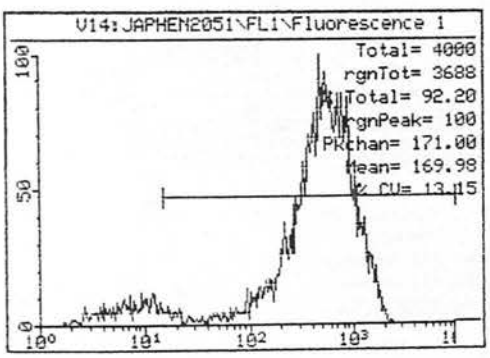
EBU141



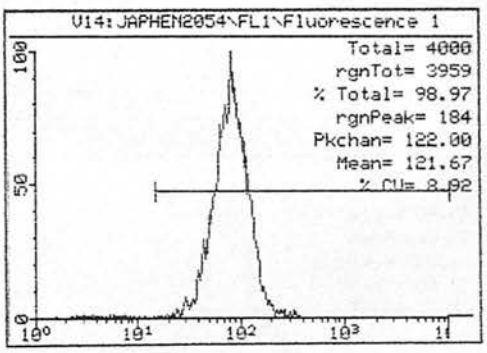
CD19



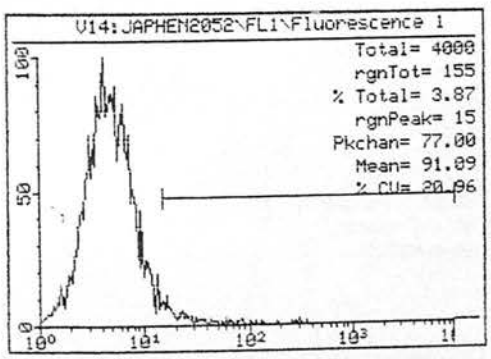
EBU65



CD5



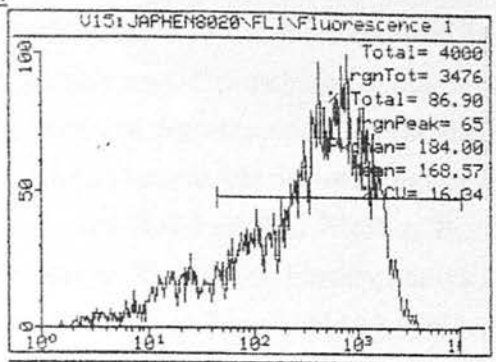
OKB4



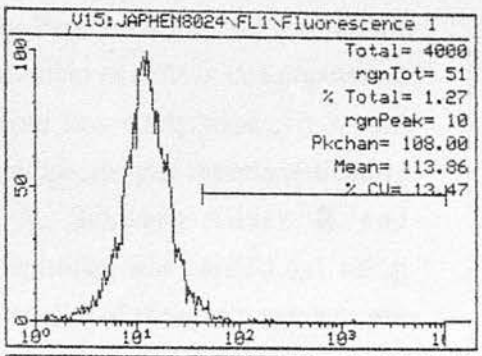
3.1.3(c) Effect of Neuraminidase on CDw75 Expression in CLL Patients.

Patient 17.

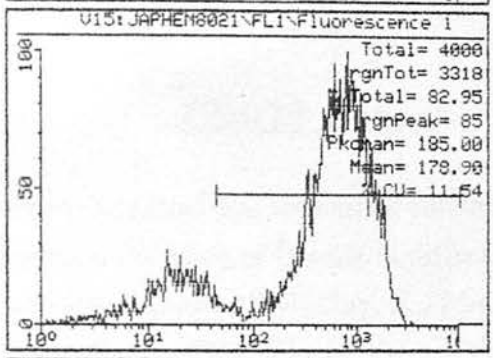
HH2



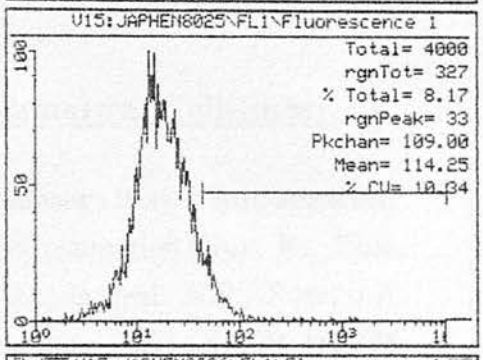
HH2 + Neur



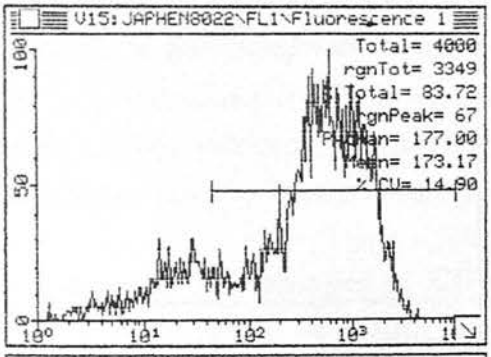
EBU141



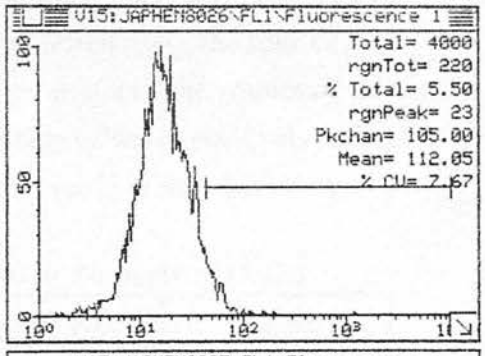
EBU141 + Neur



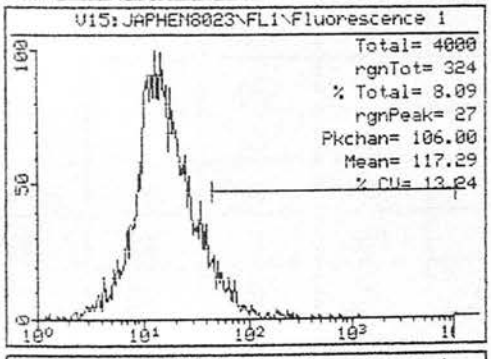
EBU65



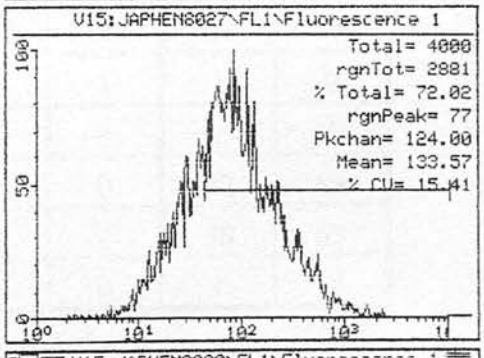
EBU65 + Neur



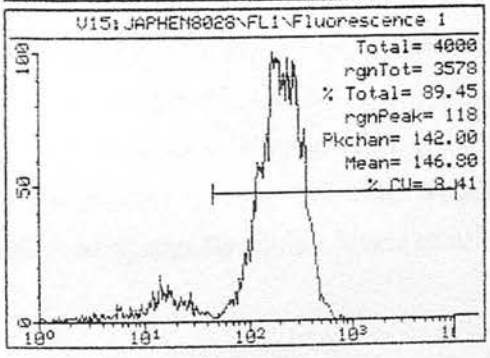
OKB4



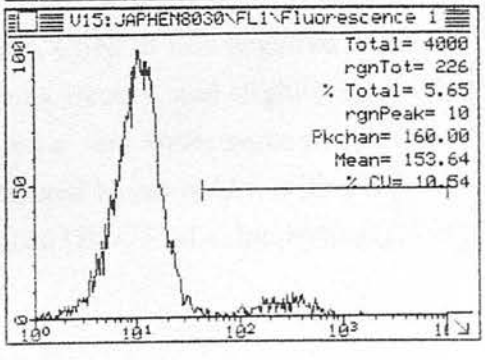
OKB4 + Neur



CD19



CD2



the epitope recognised by OKB4 is masked by sialic acid, and may even be the non-sialylated version of the HH2 epitope. In contrast, HH2, EBU-141 and EBU-65 binding was completely abolished by neuraminidase treatment, confirming that sialic acid forms a major part of these epitopes. In addition to B-cell staining, all four mAbs dimly stained a small population of non-B-cells. The number of cells in this population varied between patients and with the mAb used. From this study alone, it would appear that all four mAbs do not recognise the same epitope, despite reports indicating that they do (Dorken, B., Moller, P., Pezzutto, A., Schwartz-Albiez, R. and Moldenhauer, G., 1989). Further analysis of these epitopes was carried out using tunicamycin - an inhibitor of N-linked glycosylation. Results of these experiments are given in section 3.2.

3.1.3 CDw75 Expression on Namalwa Cell-lines.

It has been suggested that the variant sublines of the Namalwa Burkitt lymphoma cell-line are representative of B-cells at different stages of maturation.(Guy, K., Ross, J.A. and Steel, C.M., 1989; Guy, K., Middleton, P.G., Bansal, N.S., Ross, J.A. and Steel, C.M., 1990) Five of the six available sublines (NK, CSN/70, IPN/45, PNT & KN2 - in increasing order of maturation) were tested using the four CDw75 mAb on four occasions throughout the duration of the project. The results of flow cytometric analysis were consistent, and typical percentage values of positively stained cells both before and after neuraminidase treatment are given in table 3.1.2 below.

Table 3.1.2 Percentages of CDw75 Positive Namalwa Cells.

Cell Line	HH2		EBU-141		EBU-65		OKB4	
	-N	+N	-N	+N	-N	+N	-N	+N
NK	ND	ND	1	1	1	1	1	9
CSN/70	3	0	0	0	0	0	2	17
IPN/45	79	0	25	1	16	0	42	88
PNT	78	2	40	2	21	1	38	69
KN2	10	0	0	0	0	0	1	16

NK cells did not bind any of the CDw75 mAbs tested. CSN/70 was negative with EBU-141 and EBU-65. OKB4 only stained a few cells weakly, and slightly more after neuraminidase treatment. HH2 also dimly stained a very small percentage of CSN/70 cells. IPN/45 and PNT cells were brightly stained by all mAbs, with PNT cells expressing slightly higher levels of all epitopes than IPN/45 cells. Interestingly,

OKB4 bound to both sublines in significant amounts even prior to neuraminidase treatment, although binding was substantially increased after treatment. This suggests that the epitope may be only partially shielded by sialic acid on these cells, perhaps because it is expressed at such high levels. KN2 cells were found to express very low levels of the HH2 epitope and the sialylated OKB4 epitope (only detectable after neuraminidase treatment). Neither EBU-141 nor EBU-65 was found to bind to this cell-line.

CSN/70 cells do not express membrane Ig, whereas both IPN/45 and PNT cells do express this, but do not secrete Ig. KN2, the most mature subline not only expresses surface Ig, but also secretes it, mimicking mature B-cells in an activated state. NK cells are thought to be even less differentiated than CSN/70 cells(Guy, K., Middleton, P.G., Bansal, N.S., Ross, J.A. and Steel, C.M., 1990). CDw75 therefore seems to be expressed mostly on resting, mature B-cells which synthesise membrane Ig but do not secrete it, but not on immature non-Ig-expressing B-cells or on activated Ig-secreting cells. These observed cellular expression patterns correlate with those described previously at the Fourth International Leucocyte Typing Workshop(Dorken, B., Moller, P., Pezzutto, A., Schwartz-Albiez, R. and Moldenhauer, G., 1989).

3.2 Carbohydrate Structure of CDw75.

As discussed above, it is clear that the epitopes of CDw75 recognised by the mAbs HH2, EBU-141 and EBU-65 contain sialic acid, and that the OKB4 epitope is masked by sialic acid. It has also been shown previously that the epitope recognised by LN-1 is sialylated(Epstein, A.L., Marder, R.J., Winter, J.N. and Fox, R.I., 1984). In order to discover more about the carbohydrate structure of the epitopes, I attempted to determine if this was N- or O-linked carbohydrate, and if the OKB4 epitope was also composed of non-sialylated N- or O-linked carbohydrate. This was done by inhibition of synthesis of N-linked carbohydrate using tunicamycin. Tunicamycin is an antibiotic which blocks N-linked glycosylation of proteins and lipids by inhibiting the enzyme UDP-GlcNAc:dolichylphosphate-GlcNAc-1-phosphate transferase. This enzyme catalyses the first step of N-linked glycosylation - the transfer of GlcNAc-1-phosphate from UDP-N-acetylglucosamine to dolichyl phosphate to form N-acetylglucosamine pyrophosphoryl dolichol(Tkacz, J.S. and Lampen, J.O., 1975; Duksin, D. and Mahoney, W.C., 1982; Humphries, M.J., Matsumoto, K., White, S.L. and Olden, K., 1986; Broquet, P., George, P., Geoffroy, J., Reboul, P. and Louisot, P., 1991).

I also attempted to determine if the type of neuraminidase used to remove sialic acid had any effect on antigen recognition, as neuraminidase from *Vibrio cholerae* is reported to remove only terminal sialic acid residues, whilst neuraminidase from *Clostridium perfringens* removes both terminal and internal residues (Conzelmann, A. and Lefrancois, L., 1988).

3.2.1 Effect of Tunicamycin on CDw75 Expression.

PNT cells were stripped of their sialic acid by neuraminidase treatment and then cultured in varying concentrations of tunicamycin dissolved in normal RPMI for varying lengths of time with or without monensin (an inhibitor of sialyltransferase (Waibel, R., O'Hara, C.J. and Stahel, R.A., 1991)). This experiment was repeated on eight occasions with slight variations, producing consistently repeatable results (results not shown). It was found that tunicamycin effectively inhibited synthesis of the CDw75 epitopes recognised by all four available mAb.

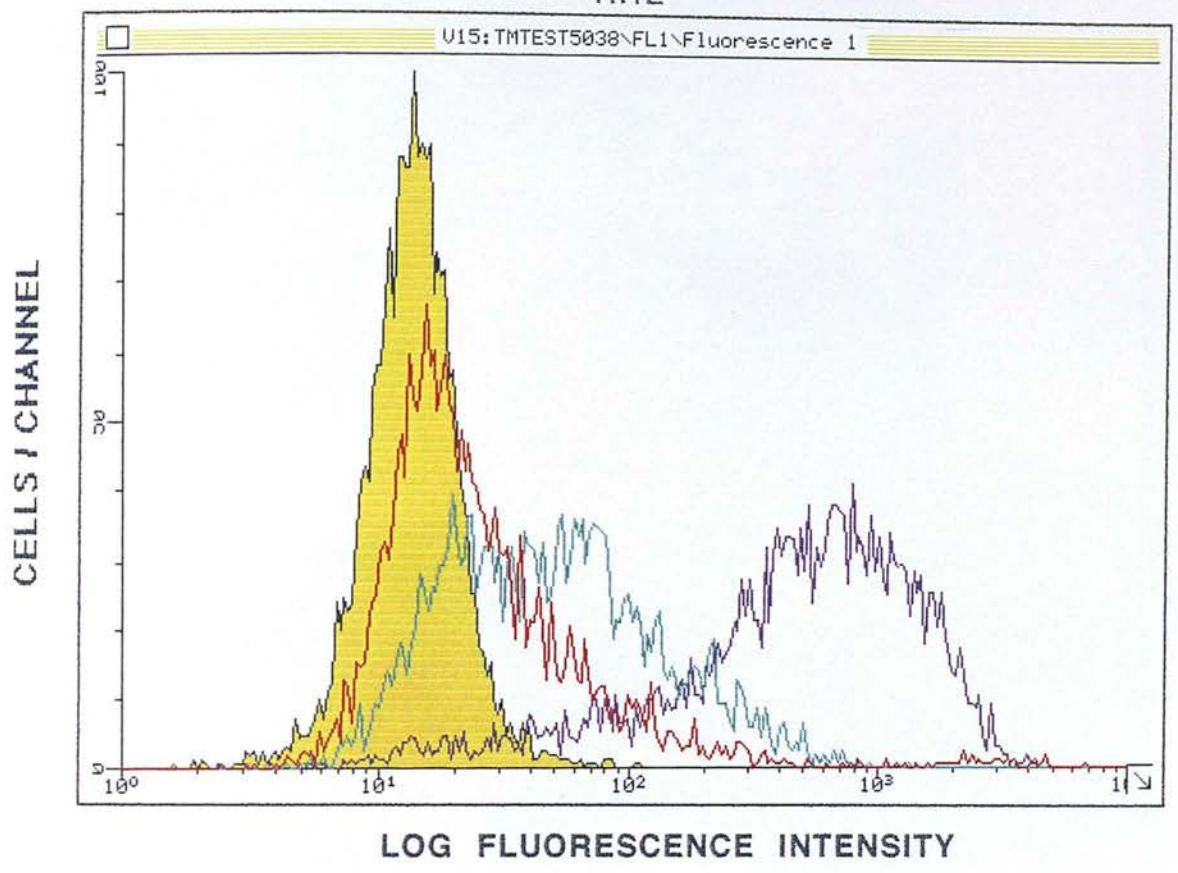
A typical example of data is given in figure 3.2.1. In this experiment, PNT cells were cultured in 20μg/ml tunicamycin + 2 μg/ml monensin. The yellow histograms represent the fluorescence levels of the negative control - cells stained with the IgM anti-CD14 mAb VIM13. The red histograms show the fluorescence levels of neuraminidase treated cells stained with the CDw75 mAb indicated. The purple curves indicate CDw75 staining levels of neuraminidase treated cells cultured overnight in normal medium after treatment (i.e. recovered cells). The green histograms show fluorescence levels of CDw75 stained cells after culturing in the presence of 20 μg/ml tunicamycin + 2 μg/ml monensin. Binding levels of all CDw75 mAb were visibly reduced after culturing in tunicamycin when compared to those of the control experiment (purple) in which normal glycosylation and CDw75 expression has been restored.

3.2.2 Effect of Tunicamycin Concentration.

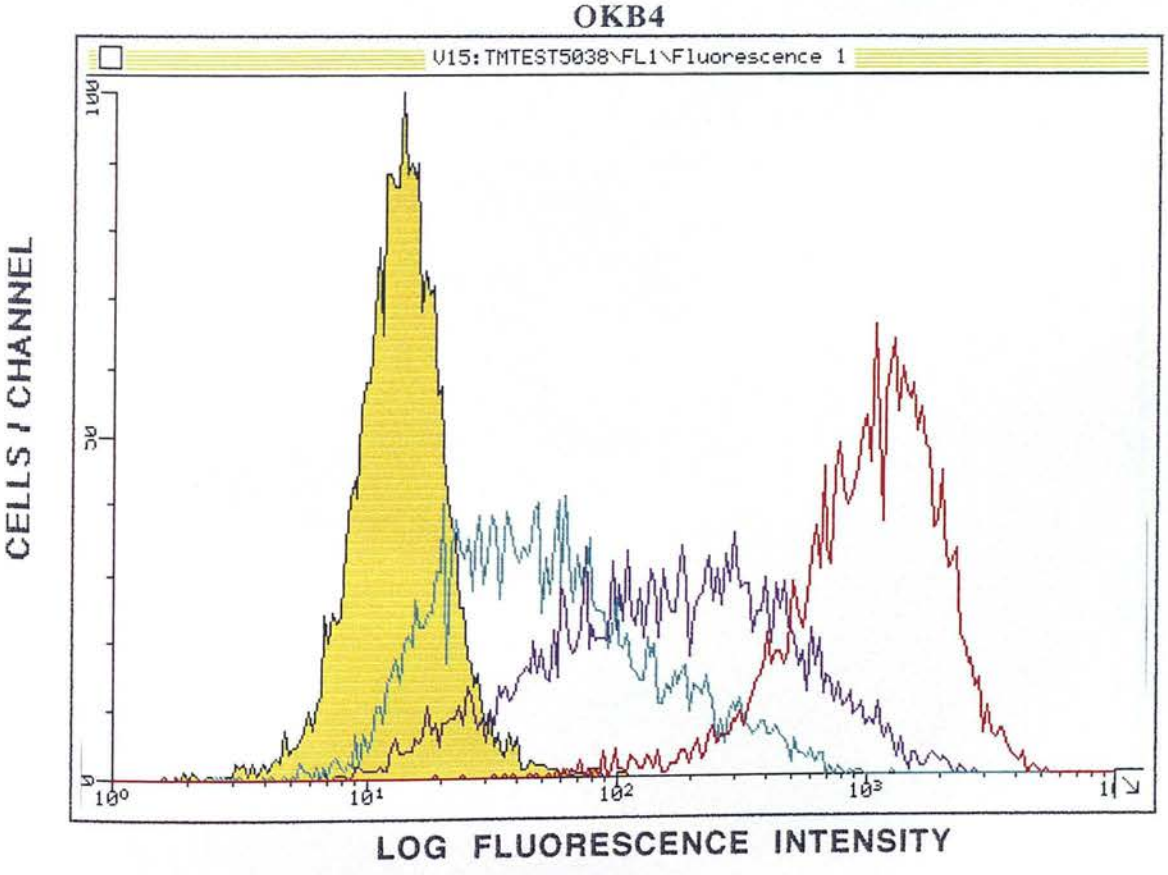
It is possible that the reduced levels of CDw75 binding observed in this experiment could be due solely to the inhibition of sialyltransferase activity by monensin. However, as demonstrated in figure 3.2.2, the tunicamycin effect is increased when increasing concentrations are used. In figure 3.2.2, the purple histograms again represent fluorescence levels of CDw75 stained control, recovered cells, and the red and green curves show CDw75 binding levels on PNT cells treated with 5 μg/ml and 20 μg/ml tunicamycin respectively. 2 μg/ml of monensin was used in all experiments.

3.2.1 Tunicamycin Inhibition of CDw75 Expression on PNT Cells.

HH2



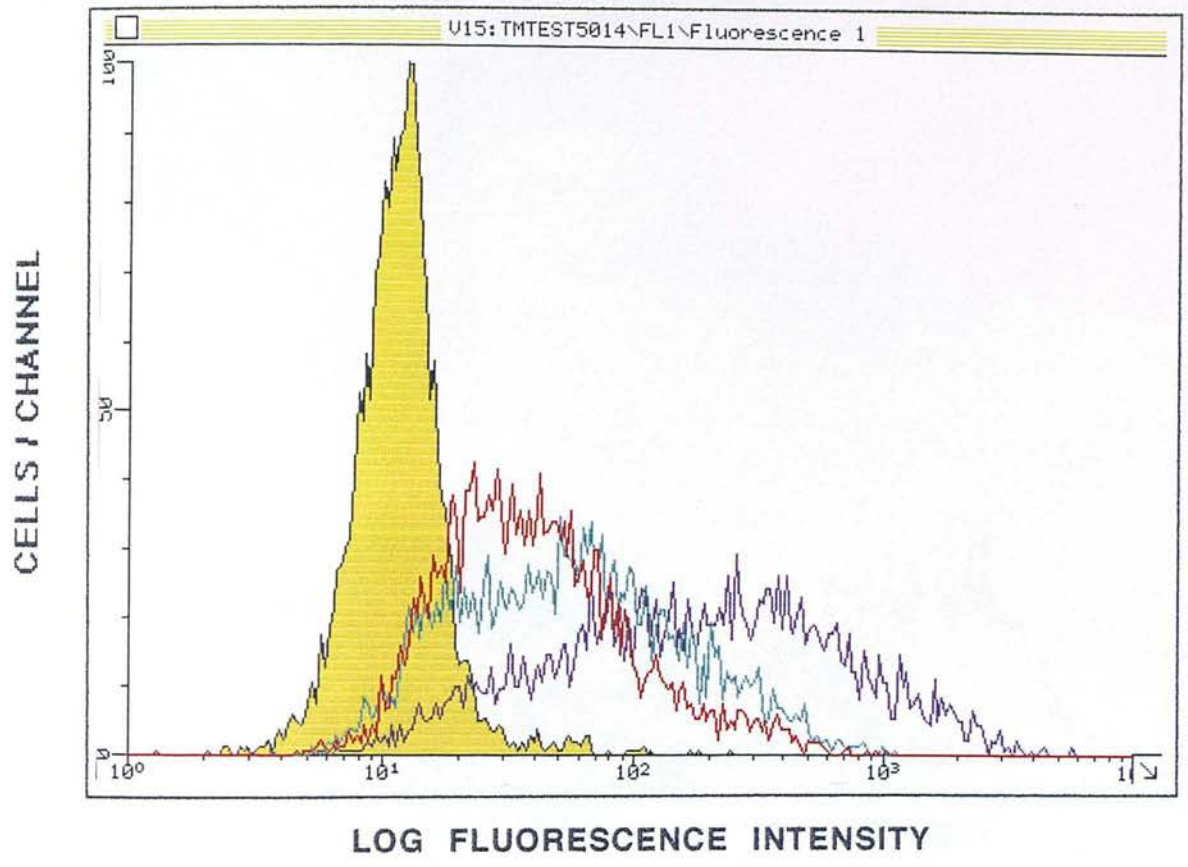
OKB4



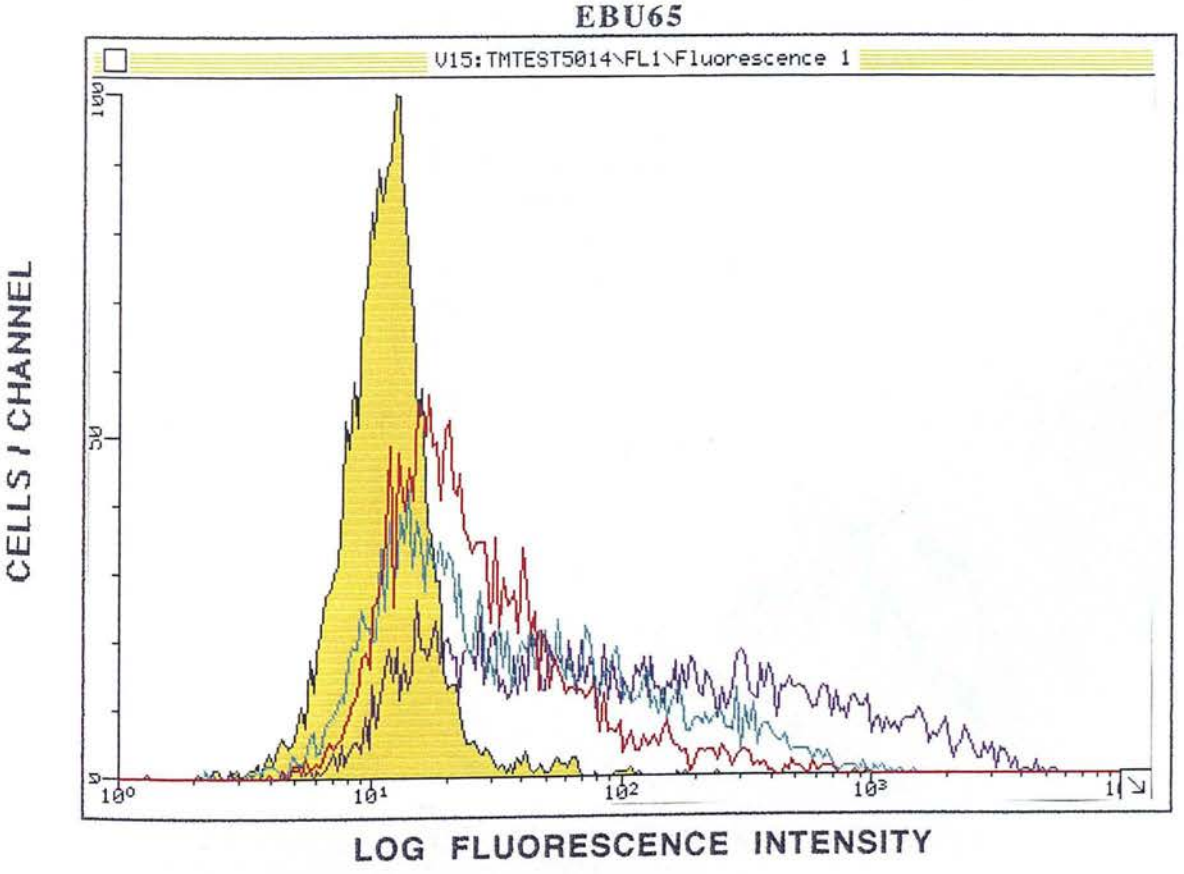
KEY:
YELLOW = ISOTYPE MATCHED NEGATIVE CONTROL
PURPLE = RE-SIALYLATED CELLS (TUNICAMYCIN = 0 μg/ml)
GREEN = CELLS TREATED WITH 20 μg/ml TUNICAMYCIN
RED = NEURAMINIDASE-TREATED CELLS

3.2.1 Tunicamycin Inhibition of CDw75 Expression on PNT Cells.

EBU141

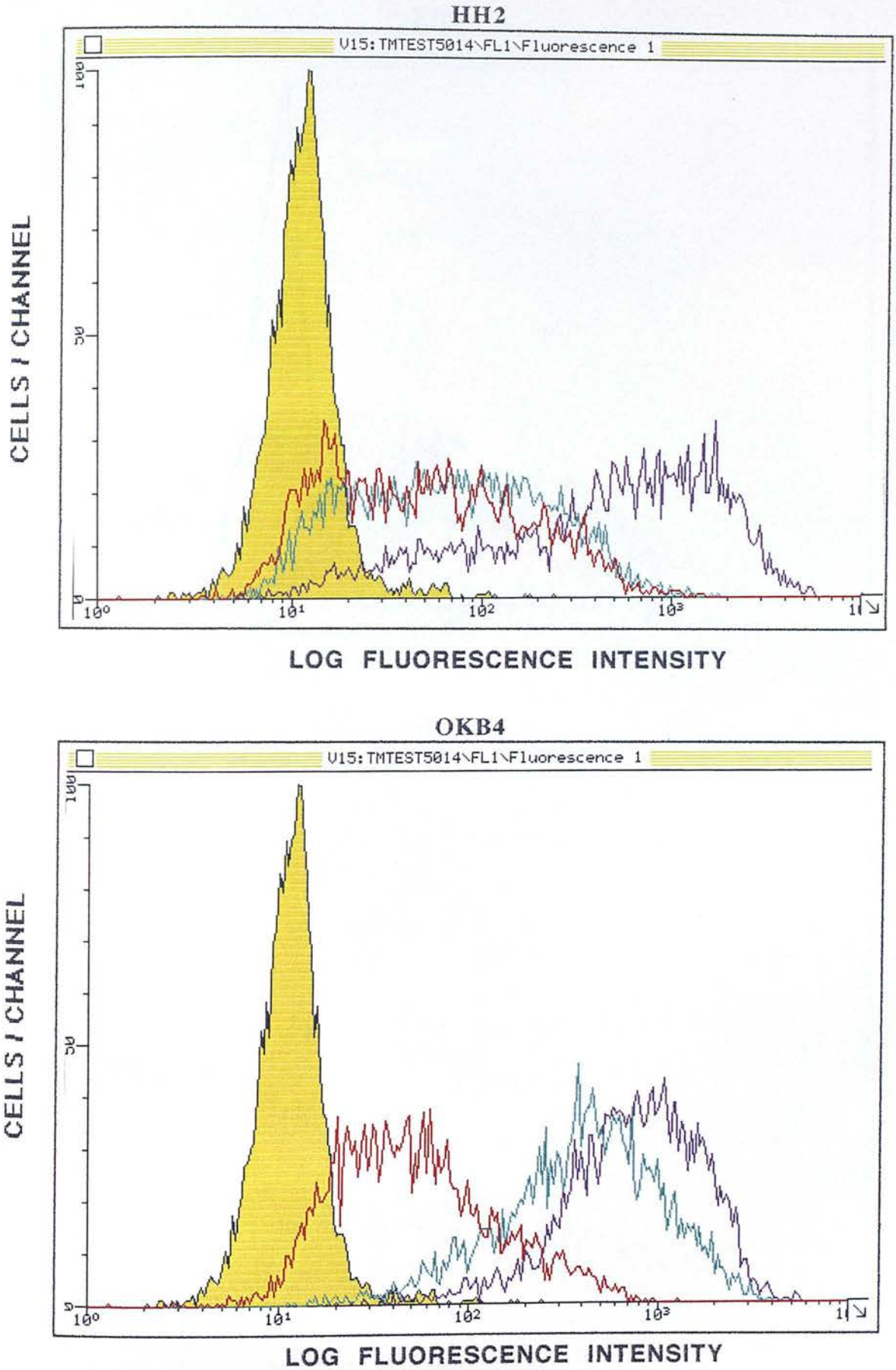


EBU65



KEY:
YELLOW = ISOTYPE MATCHED NEGATIVE CONTROL
PURPLE = RE-SIALYLATED CELLS (TUNICAMYCIN = 0 μg/ml)
GREEN = CELLS TREATED WITH 20 μg/ml TUNICAMYCIN
RED = NEURAMINIDASE-TREATED CELLS

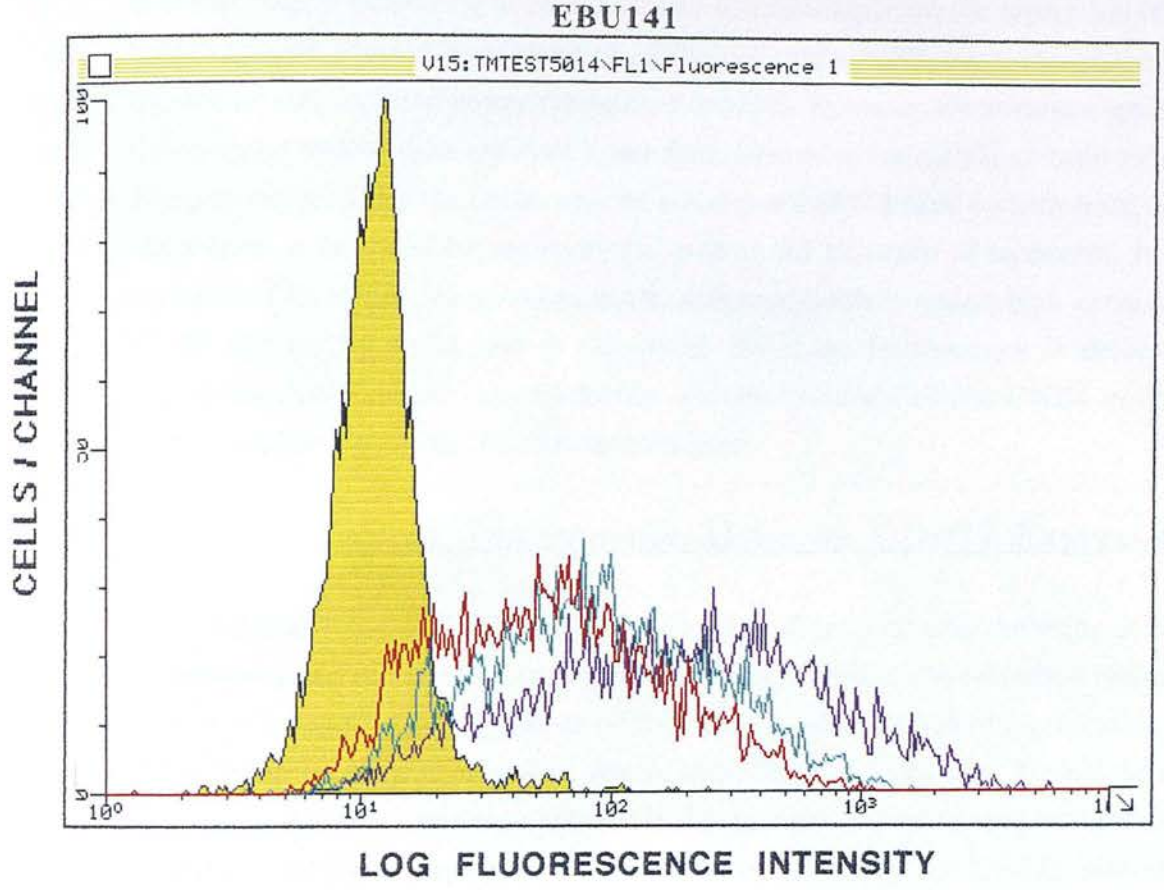
3.2.2 Effect of Tunicamycin Concentration on CDw75 Expression.

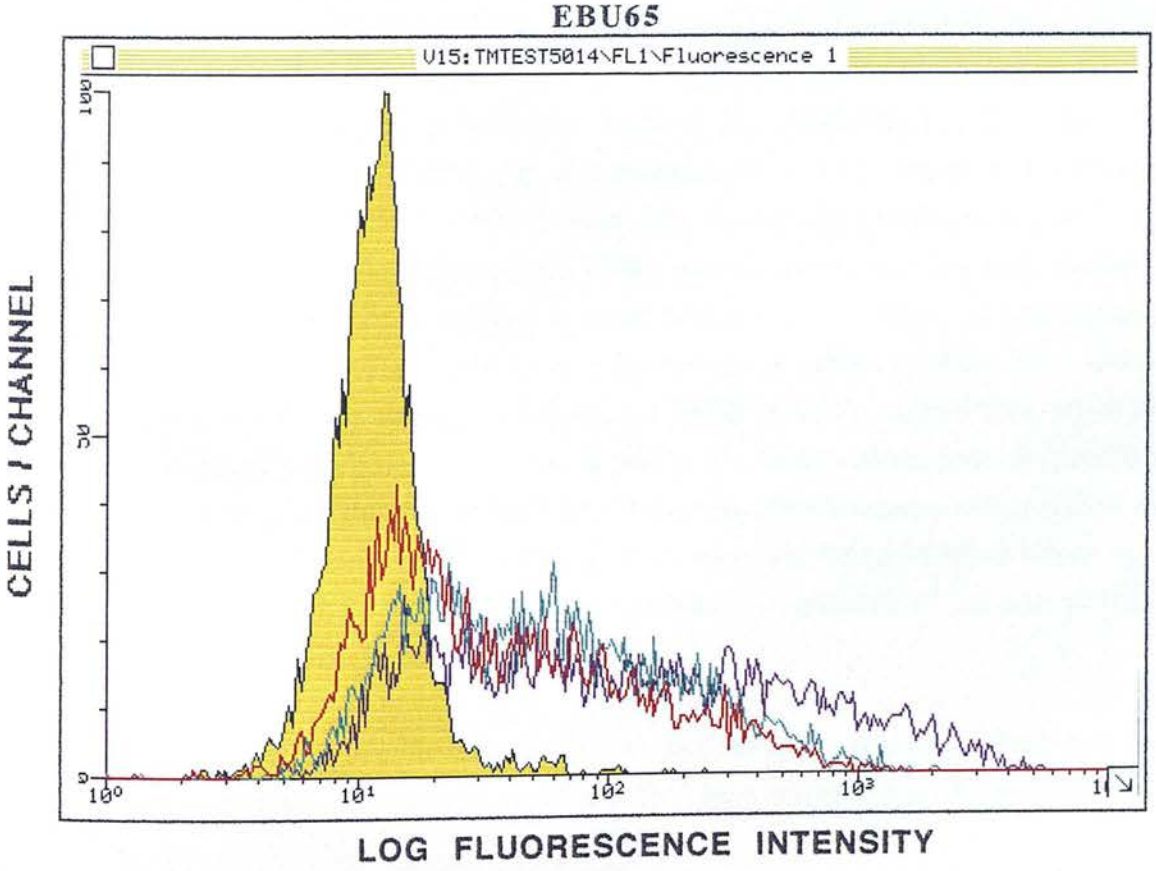


KEY:

YELLOW	=	ISOTYPE MATCHED NEGATIVE CONTROL
PURPLE	=	RE-SIALYLATED CELLS (TUNICAMYCIN = 0 μg/ml)
GREEN	=	CELLS TREATED WITH 5 μg/ml TUNICAMYCIN
RED	=	CELLS TREATED WITH 20 μg/ml TUNICAMYCIN

3.2.2 Effect of Tunicamycin Concentration on CDw75 Expression.





KEY:
YELLOW = ISOTYPE MATCHED NEGATIVE CONTROL
PURPLE = RE-SIALYLATED CELLS (TUNICAMYCIN = 0 μg/ml)
GREEN = CELLS TREATED WITH 5 μg/ml TUNICAMYCIN
RED = CELLS TREATED WITH 20 μg/ml TUNICAMYCIN

It is particularly interesting to note the effect of tunicamycin on the expression of the OKB4 epitope. Fluorescence levels of control cells are reduced due to masking of the epitope by sialylation of newly synthesised epitopes. However, fluorescence levels of tunicamycin treated cells are even lower than those of the re-sialylated control cells. The data suggests that the OKB4 epitope is composed of N-linked carbohydrate, as its expression is inhibited by tunicamycin, even in the presence of monensin. In the presence of monensin, we would expect fluorescence levels to remain high as masking of the epitope by sialic acid is prevented. However, fluorescence is decreased indicating that tunicamycin effectively inhibits synthesis of the OKB4 epitope, suggesting that it contains N-linked carbohydrate.

3.2.3 Effect of Tunicamycin Alone on CDw75 Expression.

HH2, EBU-141 and EBU-65 binding levels were all reduced after culturing cells in tunicamycin and monensin. The dependence on tunicamycin concentration indicates that these epitopes are also made up of N-linked sialylated carbohydrate, and that the reduction in staining is not solely due to the effects of monensin. To add further credence to this data, cells were cultured in the presence of tunicamycin alone without monensin, and then stained with CDw75 as before. To check the level of inhibition, cells were also stained with lectins Sambucus Nigra Agglutinin (SNA) and Maackia Amurensis Agglutinin (MAA), specific for α-2,6-linked sialic acid (only present on N-linked carbohydrate), and for α-2,3-linked sialic acid (found on both N- and O-linked carbohydrate) respectively (Shibuya, N., Goldstein, I.J., Broekaert, W.F., Nsimba-Lubaki, M., Peeters, B. and Peumans, W.J., 1987; Shibuya, N., Goldstein, I.J., Broekaert, W.F., Nsimba-Lubaki, M., Peeters, B. and Peumans, W.J., 1987; Wang, W.C. and Cummings, R.D., 1988). In one experiment, cells were treated with neuraminidase prior to staining in order to eliminate the effect of resialylation on epitope structure. The results of two experiments are given in tables 3.2.1 and 3.2.2 below. Even in the absence of monensin, CDw75 expression is inhibited, proving that all epitopes are composed at least in part of N-linked carbohydrate. In particular it should be noted that the level of OKB4 binding after treatment with 1 μg/ml and 2 μg/ml tunicamycin is unaffected by neuraminidase, providing further evidence that the reduction in OKB4 staining is due to inhibition of synthesis of the epitope and not simply to masking by sialic acid.

The percentages of cells stained with the EBU-65 mAb were particularly low in this experiment due to deterioration of the mAb. I have had to rely on the generosity of the

clone originators for all CDw75 mAb used, and it has been particularly difficult to obtain sufficient amounts of the mAbs EBU-141 and EBU-65. It would have been impossible to obtain fresh stocks of these mAbs at the stage in the project when these experiments were conducted.

Table 3.2.1Percentages of CDw75 Positive Tunicamycin-treated Cells.

	Day Zero		Tunicamycin Concentration				
mAb	control	+ neur	0µg/ml	0.25µg/ml	0.5µg/ml	1µg/ml	2µg/ml
HH2	57	1	38	17	6	2	1
OKB4	25	64	40	31	27	24	22
EBU-141	50	18	42	29	18	11	8
EBU-65	10	1	6	1	0	0	0
SNA	100	8	81	66	35	14	2
MAA	84	94	90	99	94	93	72

Table 3.2.2 Percentages of CDw75 Positive Tunicamycin-treated Cells Before and After Neuraminidase Treatment.

	Day Zero		Tunicamycin Concentration				
mAb	control	+ neur	0µg/ml	0.25µg/ml	0.5µg/ml	1µg/ml	2µg/ml
HH2	44	0	39	27	18	8	2
OKB4	16	48	35	32	29	30	22
OKB4(N)	48	48	50	45	39	32	20
EBU-141	40	9	41	34	27	29	11
EBU-65	7	1	8	4	3	2	1
SNA	89	9	94	82	67	29	15
SNA(N)	9	9	6	6	3	1	0
MAA	6	91	97	96	94	94	94

3.2.4 Comparison of Effects of Neuraminidase from *Vibrio cholerae* and *Clostridium perfringens.*

PNT cells were treated with 0.05 U/ml neuraminidase Type X from *Clostridium perfringens* as before, and with increasing concentrations of neuraminidase from *Vibrio cholerae* over the range 0 - 0.2 U/ml. Cells were then stained using the CDw75 mAb OKB4, EBU-141 and EBU-65 as well as with an isotype-matched negative control mAb (VIM13) and a known positive control mAb (4F2 - directed against an activation marker). This experiment was carried out on 2 occasions, producing similar results. Percentages of positively stained cells obtained from 1 experiment are given in table 3.2.3 below, and illustrated in figure 3.2.3.

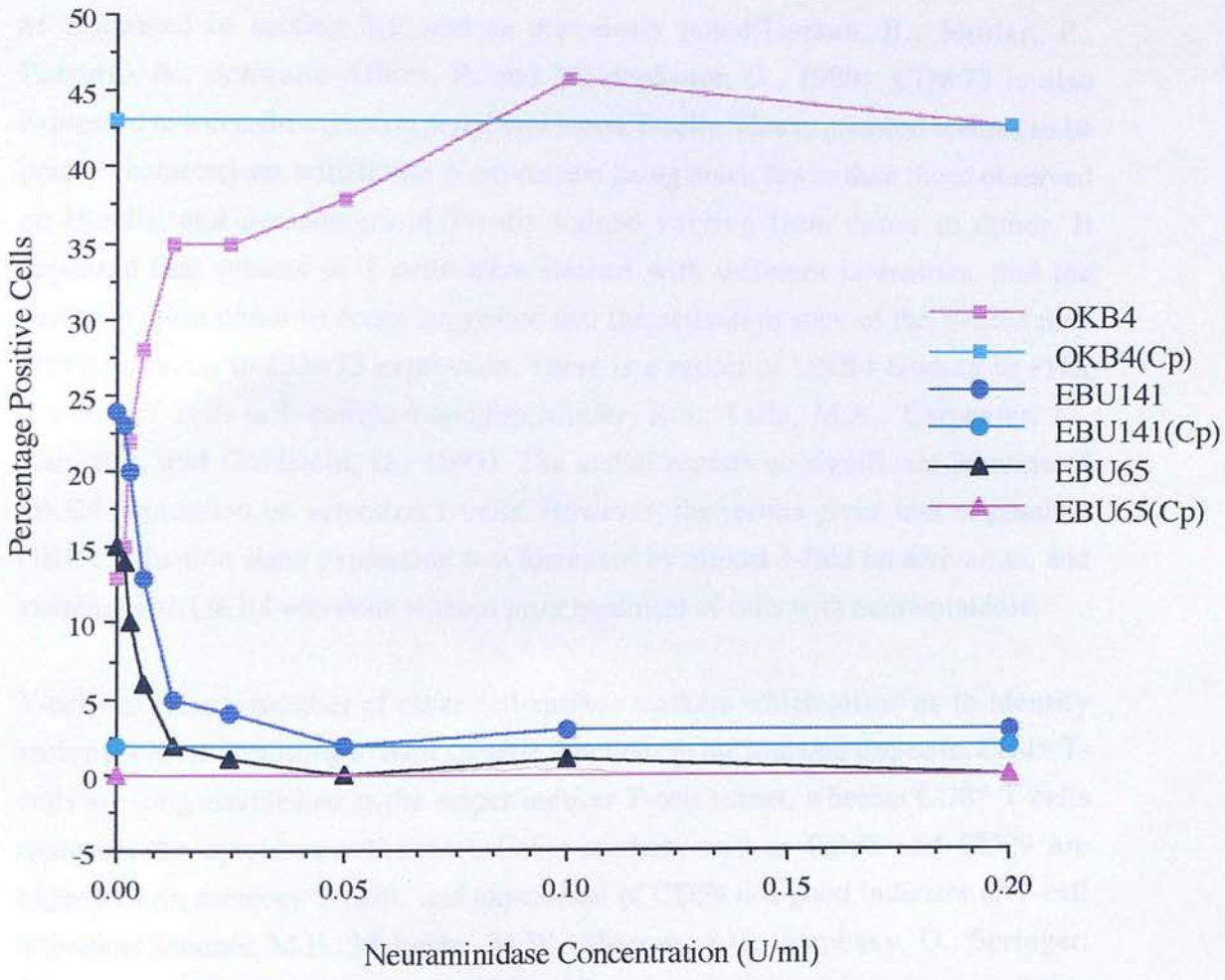
From the results it can be seen that at *Vibrio cholerae* neuraminidase concentrations greater than 0.05 U/ml, the OKB4 epitope is exposed to the same extent as with 0.05 U/ml *Clostridium perfringens* neuraminidase, and epitopes EBU-141 and EBU-65 are destroyed equally effectively by both enzymes. This indicates that the sialic acid residues involved in the EBU-141 and EBU-65 epitopes, and those which mask the OKB4 epitope are terminal sialic acid residues. Had they been internal residues, the neuraminidase from *Vibrio cholerae* would not have removed them.

Table 3.2.3 Percentages of CDw75 Positive *C.perfringens* and *V.cholerae* Neuraminidase-treated PNT Cells.

[Neuraminidase] (U/ml)	OKB4	EBU-141	EBU-65	4F2
0.016 *V.c.**	15◊	23	14	98
0.003 *V.c.*	22	20	10	98
0.006 *V.c.*	28	13	6	98
0.0125 *V.c.*	35	5	2	98
0.025 *V.c.*	35	4	1	99
0.05 *V.c.*	38	2	0	99
0.1 *V.c.*	46	3	1	99
0.2 *V.c.*	43	3	0	100
0.05 *C.p.*†	43	2	0	98
0	13	24	15	98

**V.c.* = Neuraminidase from *Vibrio cholerae.*
◊ All values are adjusted to compensate for background and non-specific binding.
†*C.p.* = Neuraminidase from *Clostridium perfringens.*

Figure 3.2.3 **Percentages of CDw75 Positive *C.perfringens* and *V.cholerae* Neuraminidase-treated PNT cells.**



3.3 CDw75 Expression on Resting and PHA-Activated Peripheral Blood T-Lymphocyte Subsets.

CDw75 expression on all mature sIg^+ B-cells has been well documented. However, as discussed in section 3.1, and as previously noted(Dorken, B., Moller, P., Pezzutto, A., Schwartz-Albiez, R. and Moldenhauer, G., 1989), CDw75 is also expressed to a certain extent on peripheral blood T-cells. This expression seemed to be poorly characterised, with levels of expression being much lower than those observed on B-cells, and percentages of T-cells stained varying from donor to donor. It appeared that subsets of T-cells were stained with different intensities, and the variation from donor to donor suggested that the activation state of the T-cells may also be a factor in CDw75 expression. There is a report of OKB4 binding to PHA activated T-cells in T-enriched samples(Mittler, R.S., Talle, M.A., Carpenter, K., Rao, P.E. and Goldstein, G., 1983). The author reports no significant increase of OKB4 expression on activated T-cells. However, the results given hint at possible OKB4 induction since expression was increased by almost 3-fold on activation, and staining with OKB4 was done without prior treatment of cells with neuraminidase.

T-cells express a number of other cell-surface markers which allow us to identify various subsets according to their specific functions in the immune response. $CD4^+$ T-cells are long established as the helper/inducer T-cell subset, whereas $CD8^+$ T-cells represent the cytotoxic cell subset. Other markers such as CD58 and CD29 are expressed on memory T-cells, and expression of CD54 is a good indicator of T-cell activation(Sanders, M.E., Makgoba, M.W., Sharrow, S.O., Stephany, D., Springer, T.A., Young, H.A. and Shaw, S., 1988). More recently it has become apparent that different isoforms of CD45 are expressed on T-cells at different stages of maturation. Naive T-cells initially express only CD45RA, but when stimulated with PHA or any soluble antigen, cells begin to express CD45RO(Akbar, A.N., L, T., Timms, A., Beverley, P.C.L. and Janossy, G., 1988; Wallace, D.L. and Beverley, P.C.L., 1990). There is a stage at around day 3 of activation where both isoforms can be detected on the cell surface, and then eventually by day 7, only CD45RO is expressed. These $CD45RO^+$ T-cells show an increased response to the same soluble antigen with kinetics typical of a secondary response. They do not revert to CD45RA expression. This primed $CD45RO^+$ T-cell subset has therefore been identified as the memory cell subset (Smith, S.H., Brown, M.H., Rowe, D., Callard, R.E. and Beverley, P.C.L., 1986; Akbar, A.N., L, T., Timms, A., Beverley, P.C.L. and Janossy, G., 1988; Sanders, M.E., Makgoba, M.W., Sharrow, S.O., Stephany, D.,

Springer, T.A., Young, H.A. and Shaw, S., 1988; Wallace, D.L. and Beverley, P.C.L., 1990).

In this section, both resting and PHA-activated T-cells were examined for expression of each of the CDw75 epitopes and for other markers of specific T-cell subsets including those discussed above. The aims of this section were to determine if CDw75 expression is increased on T-cell activation as previously suggested(Mittler, R.S., Talle, M.A., Carpenter, K., Rao, P.E. and Goldstein, G., 1983), to discover which T-cell subsets express CDw75 epitopes, and to ascertain if each CDw75 epitope is expressed on the same T-cell subsets.

3.3.1 CDw75 Expression on Resting T-Cells

In order to identify which resting lymphocyte subpopulations were CDw75 positive, normal peripheral blood lymphocytes (PBL) were double-stained with each of the four CDw75 mAb available, and with the cell surface markers CD3, CD4, CD8 and CD19. When OKB4 was used, cells were first treated with neuraminidase to expose the epitope. In all cases studied, OKB4 binding was negligible before this treatment. Typical results from a study involving six donors are shown in figure 3.3.1. In the resting state, B-cells (CD19+ cells) express CDw75 with highest intensity. HH2 and OKB4 stain relatively few T lymphocytes in the resting state. However, a small subpopulation of T-cells express high levels of those epitopes recognised by the mAbs EBU-141 and EBU-65. The majority of these T-cells are of the CD4+ type, but a proportion of CD8+ cells also expressed these epitopes.

3.3.2 EBU-141 and EBU-65 Staining of Unstimulated T-Cells.

Further analysis of these EBU-141+ and EBU-65+ populations using markers of T-cell activation such as CD45RO, CD45RA, CD54, CD58 and CD29, indicate that these T-cells belong to the naive T-cell population rather than the memory cell population.(Akbar, A.N., L, T., Timms, A., Beverley, P.C.L. and Janossy, G., 1988; Wallace, D.L. and Beverley, P.C.L., 1990) This reinforces the findings of our previously published study which indicated that EBU-65+ T-cells belong to this naive population(Guy, K. and Andrew, J.M., 1991). Representative results from one experiment where cells were stained with each of these two CDw75 mAb and the activation markers mentioned above are illustrated in figure 3.3.2. Most EBU-141 and

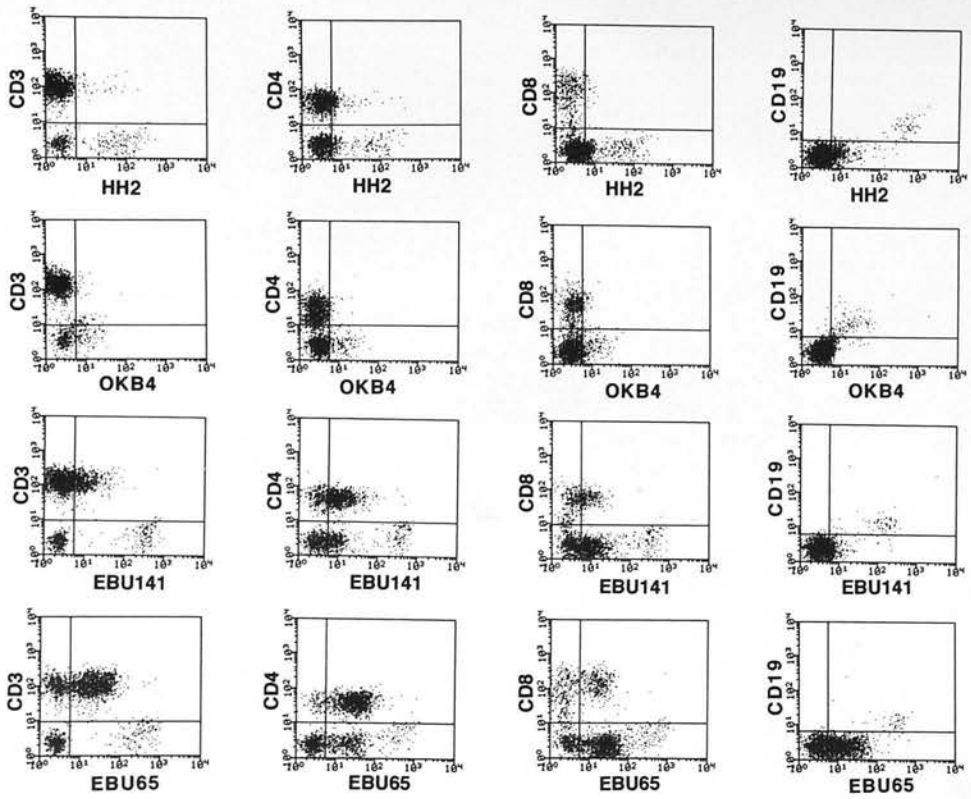


Figure 3.3.1 CDw75 staining of resting PBL.
2-colour immunofluorescence dot-plots of cells stained using FITC-anti-mouse-IgM to detect CDw75 mAbs (HH2, OKB4, EBU-141 and EBU-65), and biotinylated anti-mouse-IgG and streptavidin P.E. to detect IgG mAbs. Fluorescence is shown in log scale, and the gates superimposed indicate the limits of fluorescence intensities obtained from isotype-matched negative controls.

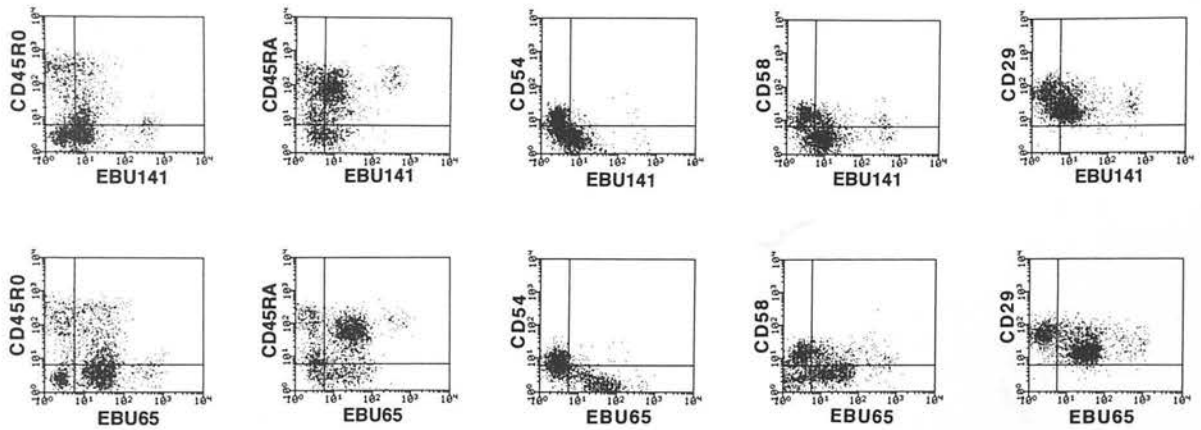


Figure 3.3.2 EBU-141 and EBU-65 staining of unstimulated T-cells.
2-colour immunofluorescence of resting T-cells stained with CDw75 and other T-cell markers of activation. EBU-141$^+$ and EBU-65$^+$ T-cells are mainly highly positive for CD45RA, and negative or weakly positive for CD45RO, CD54, CD58 & CD29.

EBU-65 positive cells are CD45RA+ and CD45RO, CD54, CD58, and CD29 negative or weakly positive - a staining pattern characteristic of naive T-cells. (Wallace, D.L. and Beverley, P.C.L., 1990)

3.3.3 CDw75 Expression on PHA-activated T-cells.

T-cells were stimulated by culturing with PHA over a three day period, and were subsequently stained with CDw75 and CD3 mAbs in order to examine changes in levels of expression of CDw75 upon T-cell activation. This experiment was repeated using PBL from eight donors. The results from one representative donor are shown in Figure 3.3.3. After stimulation for three days, the number of HH2+ and OKB4+ T-cells increased 5-10 fold, whereas binding of EBU-141 and EBU-65 mAbs was reduced. Both the number of EBU-141 and EBU-65 positive cells and the fluorescence intensity of those cells stained was reduced, with the fluorescence intensity of PHA-activated cells being approximately 10% of that of unstimulated cells.

3.3.4 CDw75 Staining of CD54$^+$ Cells.

CD54 (ICAM-1) is a marker of activated T-cells.(Sanders, M.E., Makgoba, M.W., Sharrow, S.O., Stephany, D., Springer, T.A., Young, H.A. and Shaw, S., 1988) When resting and activated T-cells are stained with CD54 and CDw75 mAbs, it is the cells with the highest levels of CD54 that express the CDw75 epitopes recognised by mAbs HH2 and OKB4 (figure 3.3.4). In contrast, although EBU-141 and EBU-65 mAb do bind to some CD54+ T-cells, they do so with much less intensity than to resting (CD54-) T-cells.

3.3.5 CDw75 staining of CD45RO$^+$ Cells

The correlation between CDw75 expression and CD45RO expression was also examined. Figure 3.3.5 illustrates typical results of PBL stained for both antigens before and after PHA activation. Before stimulation, most EBU-141 and EBU-65 positive cells are CD45RO-. However, after PHA stimulation, as most cells become CD45RO+, the intensity of EBU-141 and EBU-65 staining on these cells is greatly reduced. HH2 binding levels were, in contrast, increased on these CD45RO+ cells. Staining intensities of OKB4 on CD45RO+ cells could not be investigated as neuraminidase destroys the epitope recognised by the CD45RO mAb UCHL-1 (unpublished findings).

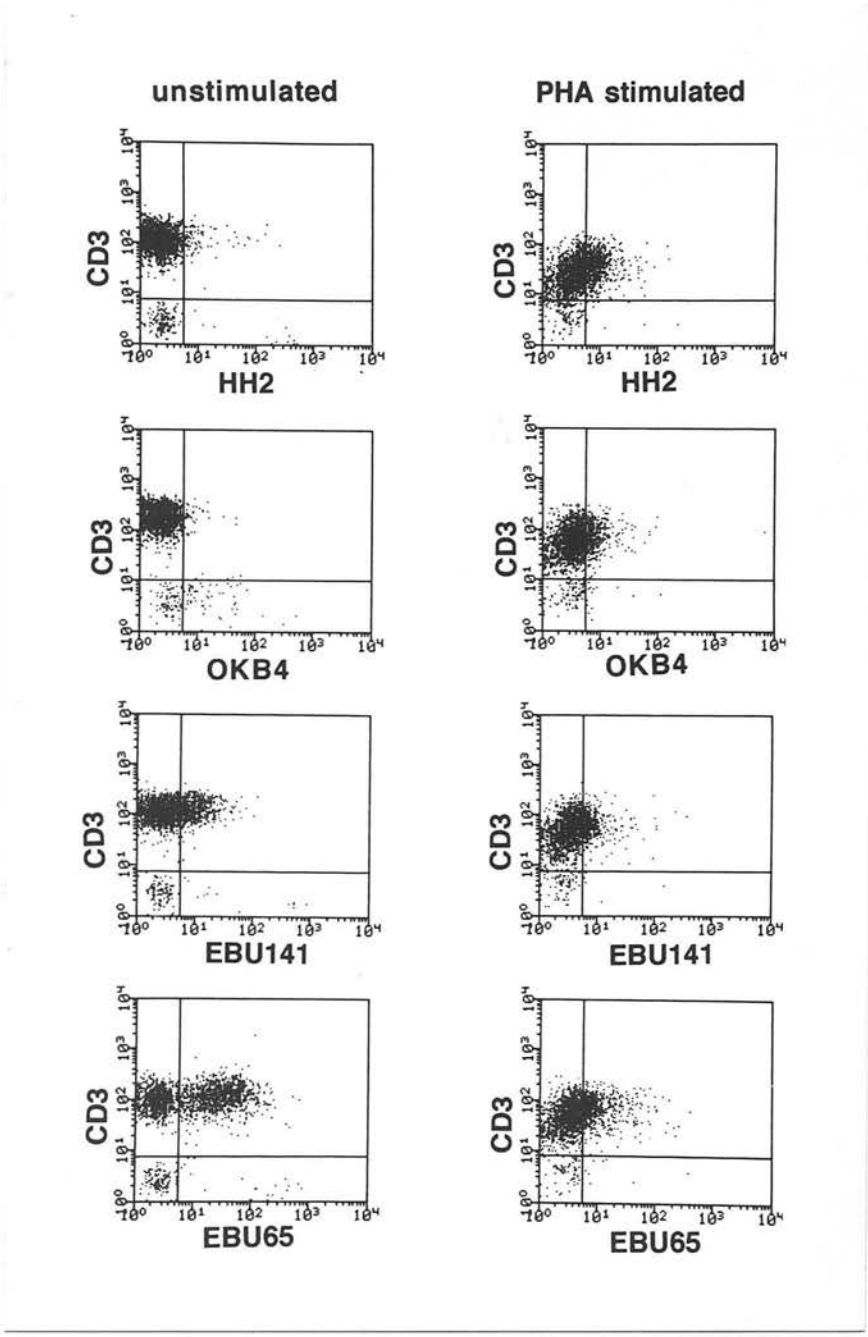


Figure 3.3.3 CDw75 staining of PHA-stimulated T-cells.
2-colour immunofluorescence of PBL before and after PHA activation. There is little or no HH2 or OKB4 staining of resting T-cells, but there is a considerable increase in the number of stained cells after activation. In contrast, EBU-141 and EBU-65 stain a large proportion of resting T-cells, but when these are activated, staining intensity and the number of cells stained are considerably reduced.

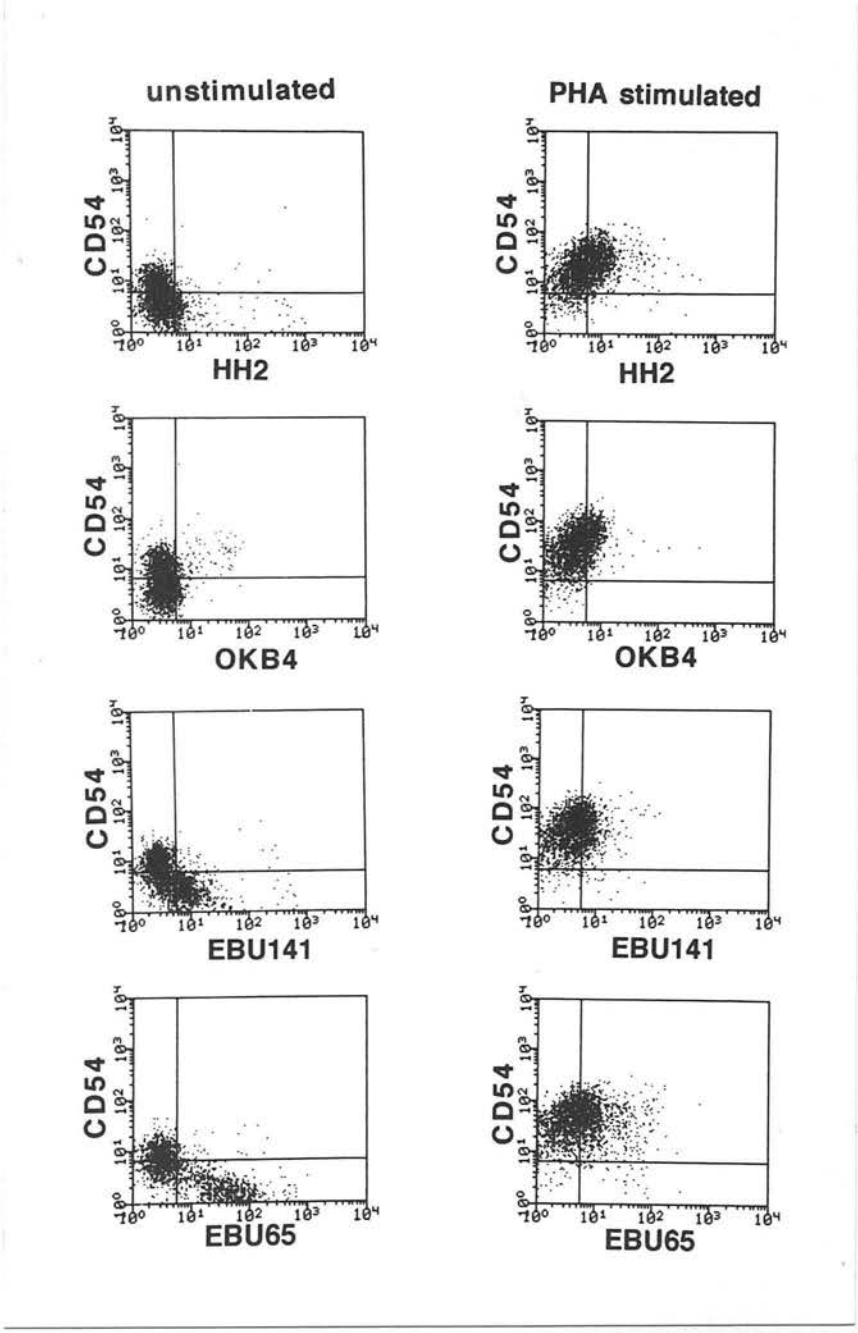


Figure 3.3.4 CDw75 staining of CD54^{+} cells.
2-colour immunofluorescence of resting and PHA-activated cells using CDw75 mAbs and anti-CD54. HH2 and OKB4 were found to weakly stain the most activated (CD54^{high+}) T-cells. Whereas EBU-141 and EBU-65 binding levels were greatly reduced upon activation.

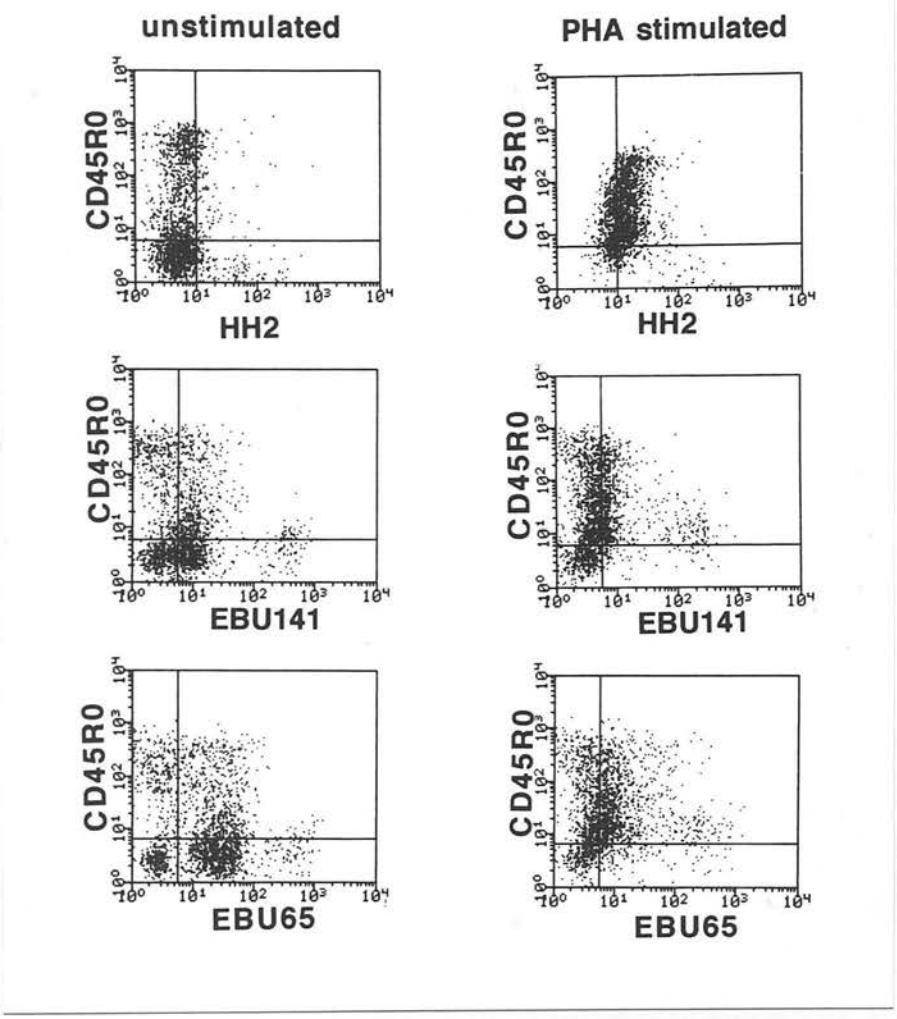


Figure 3.3.5 CDw75 staining of CD45RO$^+$ cells.
2-colour immunofluorescence of resting and PHA-activated cells using CDw75 mAbs and anti-CD54RO. HH2 binding was observed mainly on activated CD45RO$^+$ cells, whereas EBU-141 and EBU-65 staining were at highest levels on resting CD45RO$^-$ T-cells, with decreased expression of these epitopes being observed after activation (on CD45RO$^+$ cells).

3.4 Discussion.

As previously mentioned, CDw75 has been detected mainly on the surface of mature B-cells expressing surface Ig and on B-cell leukaemias and lymphomas derived from mature B-cells(Mittler, R.S., Talle, M.A., Carpenter, K., Rao, P.E. and Goldstein, G., 1983; Epstein, A.L., Marder, R.J., Winter, J.N. and Fox, R.I., 1984; Smeland, E., Funderud, S., Ruud, E., Blomhoff, H.K. and T, G., 1985; Dorken, B., Moller, P., Pezzutto, A., Schwartz-Albiez, R. and Moldenhauer, G., 1989; Erikstein, B.K., Beiske, K., Smeland, E.B., Davies, C.D.L., Blomhoff, H.K. and S, F., 1989; Gramatzki, M., Burger, R., Kraus, J., Lauer, U., Rohwer, P., Eger, G., Kalden, J.R. and Henschke, F., 1991). These findings were confirmed in section 3.1 by examining CDw75 expression on normal PBL, B-CLL cells and on the Namalwa Burkitt lymphoma cell lines. CDw75 was detected at high levels on the surface on normal B-cells, and also at low levels on normal peripheral blood T-cells when stained with the mAbs EBU-141 and EBU-65. As expected, all mAbs also brightly stained PBL of patients with B-CLL, but also dimly stained additional subpopulations of non-B-cells. The percentages of these dimly stained cells varied from patient to patient, and also with the mAb used. It is likely that these cells are also peripheral blood T-cells. Of the Namalwa sublines tested, the sIg^+ sublines IPN/45 and PNT CDw75 expressed CDw75 with highest intensity. It was found however, that HH2 and OKB4 mAbs dimly stained a small percentage of CSN/70 cells which do not express surface Ig and KN2 cells which are sIg^+ but also secrete Ig. From these experiments alone, it can be seen that there are some minor differences in expression levels and tissue distribution of the four epitopes studied. It has previously been suggested that all CDw75 mAbs recognise a single epitope(Dorken, B., Moller, P., Pezzutto, A., Schwartz-Albiez, R. and Moldenhauer, G., 1989). However, these observations alone indicate that this cannot be the case. Further evidence against this theory was found by looking at individual epitope structures, and by looking at expression of each epitope on resting and stimulated T-cell subsets.

Previous studies with the mAb LN-1 have shown that this epitope of CDw75 is sialylated(Epstein, A.L., Marder, R.J., Winter, J.N. and Fox, R.I., 1984). In this chapter I have shown that the epitopes recognised by HH2, EBU-141 and EBU-65 are also sialylated as staining of $CDw75^+$ cells with these mAbs is completely abolished when cells are pre-treated with neuraminidase. In contrast, OKB4 staining of CDw75 positive cells is markedly enhanced in cells pre-treated with neuraminidase, indicating

that this epitope is not sialylated and that sialylation of this epitope actually inhibits OKB4 binding. MAb binding to all four epitopes was also compared after treatment of cells with two different types of neuraminidase from *Clostridium perfringens* and *Vibrio cholerae*. It was thus shown that the sialic acid residues involved in all four epitopes are terminal sialic acid residues connected to the very edges of the glycosidic groups. In addition, inhibition of N-linked glycosylation by treatment of cells with tunicamycin also decreased binding levels of all four mAbs. It was therefore surmised that all four epitopes contain N-linked carbohydrate.

These experiments were carried out at a stage in the project prior to the discovery that CDw75 was not in fact a cell-surface sialyltransferase(Munro, S., Bast, B.J., Colley, K.J. and Tedder, T.F., 1992). Knowing now that CDw75 expression is dependant on the activity of the enzyme α-2,6-sialyltransferase, it would seem logical that each epitope contains N-linked carbohydrate as this enzyme specifically sialylates terminal galactose residues of N-linked carbohydrate, and does not act on O-linked carbohydrate groups. The issue of the epitope recognised by OKB4 however remains a puzzle, as although this epitope is composed of N-linked carbohydrate, its sialylation prevents recognition by this mAb. It seems strange that an epitope which is masked by the activity of sialyltransferases should be dependent on the activity of α-2,6-sialyltransferase for its expression. It has however been reported that COS cells only express the OKB4 epitope once transfected with the gene for α-2,6-sialyltransferase(Stamenkovic, I., Asheim, H.C., Deggerdal, A., Blomhoff, H.K., Smeland, E.B. and Funderud, S., 1990).

From the work carried out so far, it would appear that the CDw75 story is by no means a simple one, and that the "w" status is more than justified. Examination of T-cell subsets only proved to complicate the picture yet further, with different epitopes being upregulated and downregulated by the same stimulus. From dual staining experiments, it was shown that EBU-141 and EBU-65 dimly stain a large proportion of resting T-cells which were mostly of the $CD4^+$ type. The size of the T-cell subpopulation varied from donor to donor, and this was established to be influenced by the activation state of the T-cells. In unstimulated samples of normal PBL, EBU-141 and EBU-65 were found to stain $CD45RA^+$ T-cells i.e. naive unstimulated T-cells. Binding of HH2 and OKB4 mAbs to this T-cell population was found to be negligible. However, after stimulating cells with PHA, the expression pattern was very different. After stimulation with PHA, this $CD45RA^+$ T-cell population begins to lose CD45RA from the cell surface and start to express CD45RO(Akbar, A.N., L, T.,

Timms, A., Beverley, P.C.L. and Janossy, G., 1988). During this activation, the intensity of staining and the number of cells stained with EBU-141 and EBU-65 is reduced and that of HH2 and OKB4 is increased to levels similar to those of EBU-141 and EBU-65. Dual staining experiments showed that the $HH2^+$ cells are $CD45RO^+$. It was not possible to determine if the $OKB4^+$ cells also expressed CD45RO as neuraminidase treatment also destroys the CD45RO epitope recognised by UCHL-1. These $CD45RO^+$ and $HH2^+$ cells also express high levels of CD54 which confirms the theory that HH2 binds to activated T-cells. Also, $CD45RO^+$ T-cells expressed much lower levels of EBU-141 and EBU-65 than the unstimulated $CD45RA^+$ cells. Further study of these T-cell subsets over a longer period of stimulation would be needed in order to assess exactly which T-cell subsets express which epitopes of CDw75 and how this expression fits into the T-cell response to antigenic challenge, but it can be seen that HH2 and OKB4 epitopes are preferentially expressed on activated T-cells whereas the epitopes recognised by EBU-141 and EBU-65 are preferentially expressed on unchallenged naive T-cells. It may even be possible that these differences in expression separate the naive T-cell population from the memory T-cell population in a similar manner to the isoforms of CD45.

The results of this study clearly indicate that CDw75 is not a single antigen. Some forms are preferentially expressed by different cells and in different states of differentiation and activation on B-cells and T-cells. Although all four mAbs tested preferentially stained mature sIg^+ B-cells, HH2 and OKB4 also stained some pre-B-cell-like cells and some more differentiated B-cells which not only express sIg but also secrete it. In addition, HH2 and OKB4 do not stain resting T-cells, whereas EBU-141 and EBU-65 stain these cells very brightly. After PHA activation however, all four mAbs appear to stain T-cells with similar intensity indicating that an induction of the HH2 and OKB4 epitopes and a reduction in the expression of EBU-141 and EBU-65 epitopes is produced by the same stimulus. In addition, the physical structure of the epitopes of CDw75 are not identical, as HH2, EBU-141, EBU-65 and LN-1 are sialylated carbohydrates, whereas the OKB4 epitope is masked when sialylated. It is possible that this epitope does not belong in the cluster at all, and this will be discussed at length in the ensuing chapters.

CHAPTER 4

Tissue Distribution of CDw75.

4.1 A comparison of CDw75 distribution in solid-tumour and normal tissue sections.

As discussed in chapter 1, the oligosaccharide portion of glycoproteins and glycolipids is thought to play a very important role in the processes of invasive tumour growth and metastasis. Metastasis involves the dissemination of tumour cells into distant organs and is a complex and multi-step process. This requires the release of cells from the primary tumour, invasion into blood vessels, transport and survival of blood-borne tumour cells, homing to preferred organs, extravasion from capillaries and infiltration of the target tissue(Smets, L.A. and Van Beek, W.P., 1984). Many observations are consistent with the idea that changes in the glycosylation of cell-surface molecules may account for the ability of tumour cells to implant and metastasise. Correlations have been shown to exist between levels of fucosylation of cell-surface glycoproteins and glycolipids and malignant transformation of cells(Yamashita, K., Ohkura, T., Tachibana, Y., Takasaki, S. and Kobata, A., 1984), but it is perhaps of more importance that the occurrence of increased branching and sialylation of membrane-bound carbohydrate has also been confirmed in various solid tumour cells grown and labelled *in vivo*(Smets, L.A. and Van Beek, W.P., 1984). In addition, several groups have shown a correlation between levels of cell-surface sialylation and tumour aggression(Yogeeswaran, G. and Salk, P.L., 1981; Hakomori, S., Patterson, C.M., Nudelman, E. and Sekiguchi, K., 1983; Smets, L.A. and Van Beek, W.P., 1984; Dennis, J.W., 1986; Humphries, M.J., Matsumoto, K., White, S.L. and Olden, K., 1986; Cohen, A.M., Allalouf, D., Djaldetti, M., Weigl, K., Lehrer, N. and Levinsky, H., 1989; Dall'Olio, F., Malagolini, N., di Stefano, G., Minni, F., Marrano, D. and Serafini-Cessi, F., 1989; Francina, A., Gateau-Roesch, O., Couprie, N., Leculier, C., Col, J.F., Archimbaud, E., Campos, L., Louisot, P. and Richard, M., 1989; Springer, G.F., 1989; Bresalier, R.S., Rockwell, R.W., Dahiya, R., Duh, Q.Y. and Kim, Y.S., 1990; Easton, E.W., Bolscher, J.G.M. and van den Eijnden, D.H., 1991; Sasaki, H., Momoi, T., Yamanaka, C., Yorifuji, T., Kaji, M. and Mikawa, H., 1991; Harvey, B.E., Toth, C.A., Wagner, H.E., Steele, G.D.J. and Thomas, P., 1992; Vandamme, V., Cazlaris, H., Le Marer, N., Laudet, V., Lagrou, C., Verbert, A. and Delannoy, P., 1992). The alterations in glycosylation patterns of cell-surface glycoproteins and glycolipids are usually explained by altered levels of specific glycosyltransferases in tumour cells. It has been suggested that the same core protein on the cell surface can be differently glycosylated in different tissues and in tumour cells as compared with normal cells(Taylor-Papadimitriou, J., 1991). Some studies have indicated that

increased levels of β-1,4-galactosyltransferase(Uemura, M., Sakaguchi, T., Uejima, T., Nozawa, S. and Narimatsu, H., 1992), or alternative transcripts of this enzyme(Uejima, T., Uemura, M., Nozawa, S. and Narimatsu, H., 1992) are associated with ovarian cancer. In another study, divergent expression of different fucosyltransferases was observed in certain colon carcinomas(Stroup, G.B., Anumula, K.R., Kline, T.F. and Caltabiano, M.M., 1990). Others have also suggested that the branching enzyme N-acetylglucosamine transferase may play a crucial role in the cell surface changes associated with transformation and tumour development(Smets, L.A. and Van Beek, W.P., 1984). Blood group antigens such as GalNAcα-O (the basis of blood group A) have also been implicated in oncogenic transformations, and have even been linked with aggressive tumour behaviour(Springer, G.F., 1989; Wolf, G.T., Carey, T.E., Schmaltz, S.P., McClatchey, K.D., Poore, J., Glaser, L., Hayashida, D.J.S. and Hsu, S., 1990; Brooks, S.A. and Leathem, A.J.C., 1991). However, this antigen has also been shown to be protective in some lung cancers(Lee, J.S., Ro, J.Y., Sahin, A.A., Hong, W.K., Brown, B.W., Mountain, C.F. and Hittelman, W.N., 1991). In a number of studies, increased levels of sialyltransferase activity has been associated with cell transformation(Yamashita, K., Ohkura, T., Tachibana, Y., Takasaki, S. and Kobata, A., 1984; Cohen, A.M., Allalouf, D., Bessler, H., Djaldetti, M., Malachi, T. and Levinsky, H., 1989; Cohen, A.M., Allalouf, D., Djaldetti, M., Weigl, K., Lehrer, N. and Levinsky, H., 1989; Dall'Olio, F., Malagolini, N., di Stefano, G., Minni, F., Marrano, D. and Serafini-Cessi, F., 1989; Francina, A., Gateau-Roesch, O., Couprie, N., Leculier, C., Col, J.F., Archimbaud, E., Campos, L., Louisot, P. and Richard, M., 1989; Ledinko, N. and Fazely, F., 1989; Kanani, A., Sutherland, D.R., Fibach, E., Matta, K.L., Hindenburg, A., Brockhausen, I., Kuhns, W., Taub, R.N., van den Eijnden, D.H. and Baker, M.A., 1990; Dall'Olio, F., Malagolini, N., Di Stefano, G., Ciambella, M. and Serafini-Cessi, F., 1991; Sasaki, H., Momoi, T., Yamanaka, C., Yorifuji, T., Kaji, M. and Mikawa, H., 1991; Harvey, B.E., Toth, C.A., Wagner, H.E., Steele, G.D.J. and Thomas, P., 1992; Kobayashi, H., Terao, T. and Kawashima, Y., 1992; Vandamme, V., Cazlaris, H., Le Marer, N., Laudet, V., Lagrou, C., Verbert, A. and Delannoy, P., 1992). Many of these studies do not distinguish between the different sialyltransferases which may be involved, but other studies have specifically shown that β-galactoside-α-2,6-sialyltransferase activity is upregulated in comparison to α-2,3-sialyltransferase on metastatic cell lines(Hakomori, S., Patterson, C.M., Nudelman, E. and Sekiguchi, K., 1983; Dall'Olio, F., Malagolini, N., di Stefano, G., Minni, F., Marrano, D. and Serafini-Cessi, F., 1989; Bresalier, R.S., Rockwell, R.W., Dahiya, R., Duh, Q.Y. and Kim,

Y.S., 1990; Dall'Olio, F., Malagolini, N., Di Stefano, G., Ciambella, M. and Serafini-Cessi, F., 1991; Dall'Olio, F., Malagolini, N. and Serafini-Cessi, F., 1992; Dall'Olio, F., Malagolini, N. and Serafini-Cessi, F., 1992; Jiang, M., Passaniti, A., Penno, M.B. and Hart, G.W., 1992; Kobayashi, H., Terao, T. and Kawashima, Y., 1992) and in *ras*- transformed cells(Easton, E.W., Bolscher, J.G.M. and van den Eijnden, D.H., 1991; Vandamme, V., Cazlaris, H., Le Marer, N., Laudet, V., Lagrou, C., Verbert, A. and Delannoy, P., 1992).

As previously described, CDw75 was thought to be a cell-surface form of the enzyme β-galactoside-α-2,6-sialyltransferase which has been shown to be expressed at high levels on various transformed cell-lines of B-cell origin as discussed in chapter 3. Since sialyltransferases have also been shown to play a role in so many different types of solid tumours as discussed above, it seemed a logical step to compare CDw75 expression in a number of samples of normal and cancerous tissues. It was hoped that the CDw75 mAbs may provide some sort of prognostic marker for tumour aggressiveness in some tissues.

Table 4.1Number ofTissue samples analysed for CDw75 expression.

Lymphoid Tissues		Non-Lymphoid Tissues	
Tonsil	(1)	Liver	(11)
Lymph Node	(2)	Pancreas	(9)
Spleen	(1)	Intestine	(12)
Thymus	(1)	Breast	(4)
		Lung	(1)

Samples of various lymphoid and non-lymphoid tissues (listed in table 4.1) were stained with the four available CDw75 mAbs (HH2, OKB4, EBU-141 & EBU-65) using immunohistochemical techniques. It was aimed to stain and analyse ten sample of each tissue type (5 normal and 5 tumour). However, the number of samples analysed was restricted by availability and by the quality of the samples available.

Tissue samples were obtained from patients suffering from various forms of cancer, and others were normal samples, or apparently normal samples taken from a site distant from the cancerous tissue in the same patient. This is by no means a comprehensive comparison of CDw75 expression on normal and tumour tissue, as the study was very much limited by the quality and quantity of tissue available. However,

the results obtained in this study do serve to confirm and expand on those findings already described in the existing literature.

4.1.1 CDw75 Expression on Tonsil.

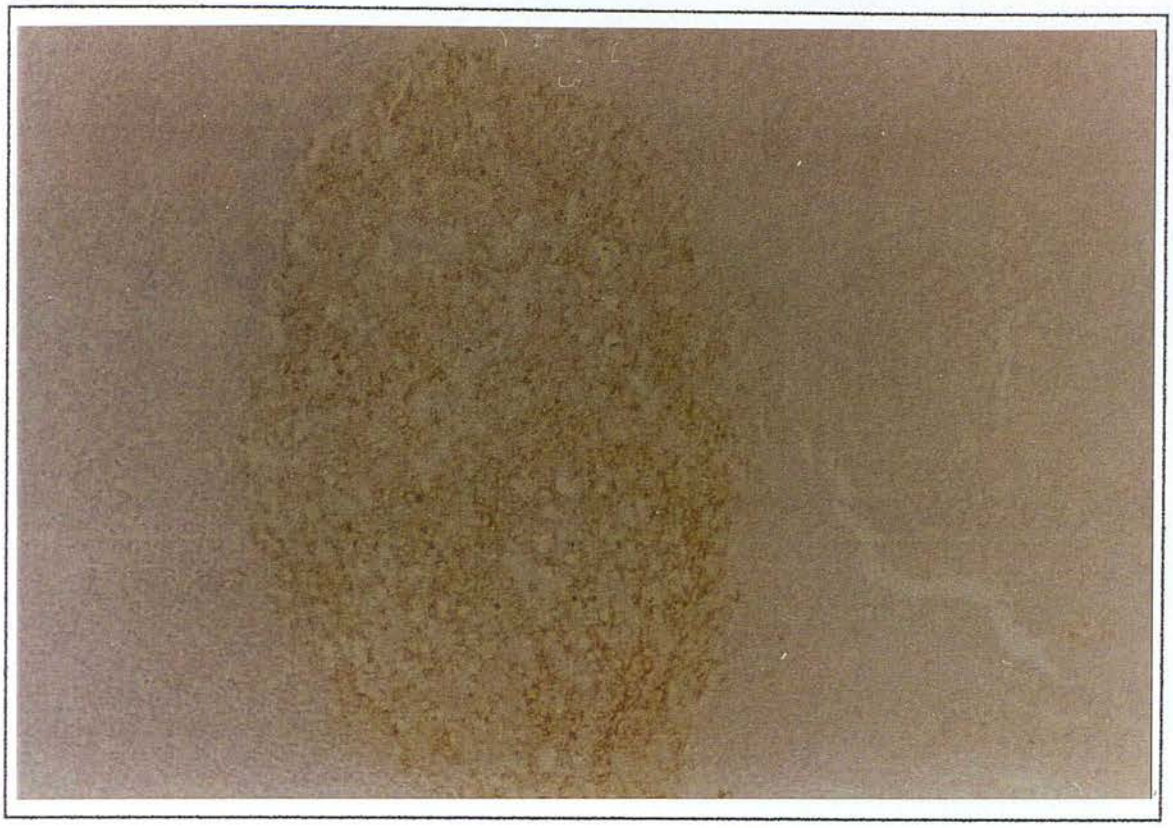
Only one tonsil sample was studied (block RL91-630). This was a normal tonsil sample, and sections of this were routinely used as positive controls when staining other tissue samples. The staining pattern observed using CDw75 mAb on this tissue was very similar to that described in the literature (Epstein, A.L., Marder, R.J., Winter, J.N. and Fox, R.I., 1984; Dorken, B., Moller, P., Pezzutto, A., Schwartz-Albiez, R. and Moldenhauer, G., 1989; Gramatzki, M., Lauer, U., Burger, R., Huber, C., Rohwer, P., Kalden, J.R. and Henschke, F., 1989). Photographs of HH2 and EBU-141 staining on this tonsil can be seen in plates 4.1.1 and 4.1.2. Both HH2 and OKB4 stained cells of the B-cell follicles with high intensity, with a staining pattern like that shown in plate 4.1.1. Both mAb also stained occasional mantle zone cells as can be seen in a higher magnification of the same section in plate 4.1.1b. As can be seen in photograph 4.1.2, EBU-141 staining indicates low levels of CDw75 expression throughout the tonsil, with higher levels of expression in the cells of the B-cell follicles where staining is very intense. EBU-65 staining was also weak and was limited to the B-cell follicles.

4.2 CDw75 Expression on Lymph Node.

Lymph node samples from two patients were studied. One (8180/89) with follicular hyperplasia, and another (16069/89) with paracortical hyperplasia. Both samples produced similar staining patterns with the four CDw75 mAbs tested.

HH2 produced a similar pattern in lymph node to that found on tonsil, with B-cell follicles in the outer cortex staining strongly and occasional mantle zone cells also positive. The other three mAbs tested did not produce any staining at all. A lymph

Photographic Plate 4.1.1



(a) HH2 staining of normal tonsil RL91-630. B-cell follicle (x 10 magnification).

(b) HH2 staining of normal tonsil RL91-630. Mantle zone (x 40 magnification).

Photographic Plate 4.1.2

(a) EBU-141 staining of normal tonsil RL91-630. B-cell follicle (x 10 magnification).

node stained with HH2 and with a pan-B-cell mAb against CD19 is shown in photograph plate 4.2.1. As can be seen in 4.2.1a, HH2 staining is confined mainly to the B-cell follicles, with very little staining of the mantle zone. This contrasts with the pattern found when the same lymph node was stained with a B-cell marker, CD19, (4.2.1b) where positive B-cells were found throughout the follicles and the surrounding mantle zones.

LN-1 has been reported to give a similar staining pattern to that observed with HH2 (Epstein, A.L., Marder, R.J., Winter, J.N. and Fox, R.I., 1984). EBU-141 and EBU-65 have also been reported to stain the normal B-cell areas in lymph node (Gramatzki, M., Burger, R., Kraus, J., Lauer, U., Rohwer, P., Eger, G., Kalden, J.R. and Henschke, F., 1991). I did not find this, but it is possible that the activity of the antibodies used had deteriorated at the stage of the project when this tissue was studied. Fresh mAb supplies were obtained for later work.

4.3 CDw75 Expression on Spleen.

Only one, sample of normal spleen (8064/87) was obtained and stained with the four CDw75 mAbs available. All four mAbs stained the marginal zone of the white pulp, and HH2 and EBU-141 also stained occasional cells in the germinal centres. i.e. CDw75 is found in the normal B-cell areas of the spleen. The T-cell areas of the spleen are clearly demonstrated in photographic plate 4.3.1b - a section of spleen stained with the T-cell marker, CD3. Photographs of OKB4- and EBU-141-stained sections are shown in plates 4.3.1a & 4.3.2. OKB4 mainly stains the area surrounding the T-cell follicles (4.3.1a) and contrasts with the CD3-stained example of the same section (4.3.1b). An example of EBU-141 staining is shown in 4.3.2a, where staining is mainly confined to the area outwith the T-cell follicles, and in higher magnification (4.3.2b) we can see occasional EBU-141 staining of follicular cells. EBU-65 produced a similar staining pattern to EBU-141, but did not stain any of the cells of the germinal centres. LN-1 has been reported to stain germinal centres of the white pulp in spleen (Epstein, A.L., Marder, R.J., Winter, J.N. and Fox, R.I., 1984).

4.4 CDw75 Expression on Thymus.

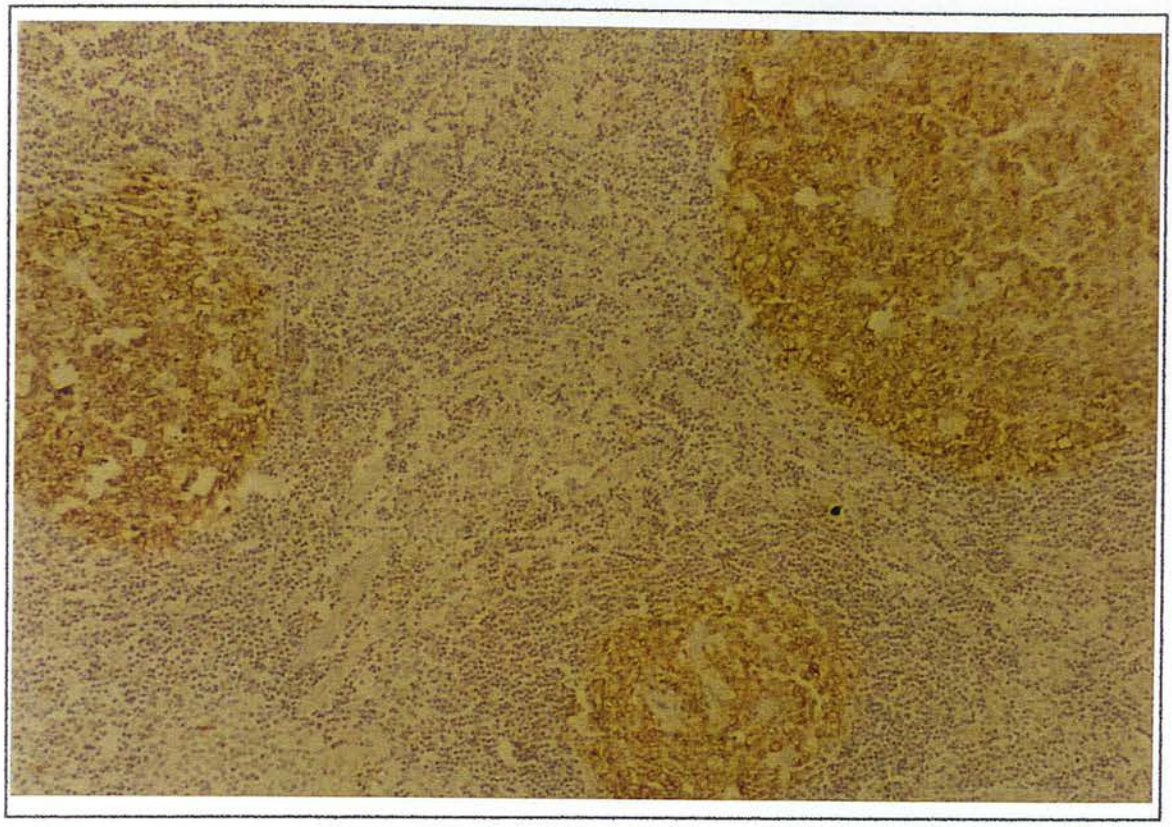
Only one sample of normal thymus was obtained (RL91-582) and stained as before. Only occasional cells and clumps of medullary thymocytes were stained with all four mAbs. The vast majority of thymocytes were CDw75 negative. Photographs of a section stained with HH2 and with the B-cell marker CD19 are shown in plate 4.4.1. CDw75 distribution in the thymus appeared to be very similar to that of B-cells, which seems to suggest that CDw75 is expressed on the B-cells of the thymus and not on the immature T-cells of the thymus. Photographs of OKB4 and EBU-141 staining of thymus are shown in plate 4.4.2. There are no other previous reports of CDw75 binding to thymocytes.

4.5 CDw75 Expression on Liver.

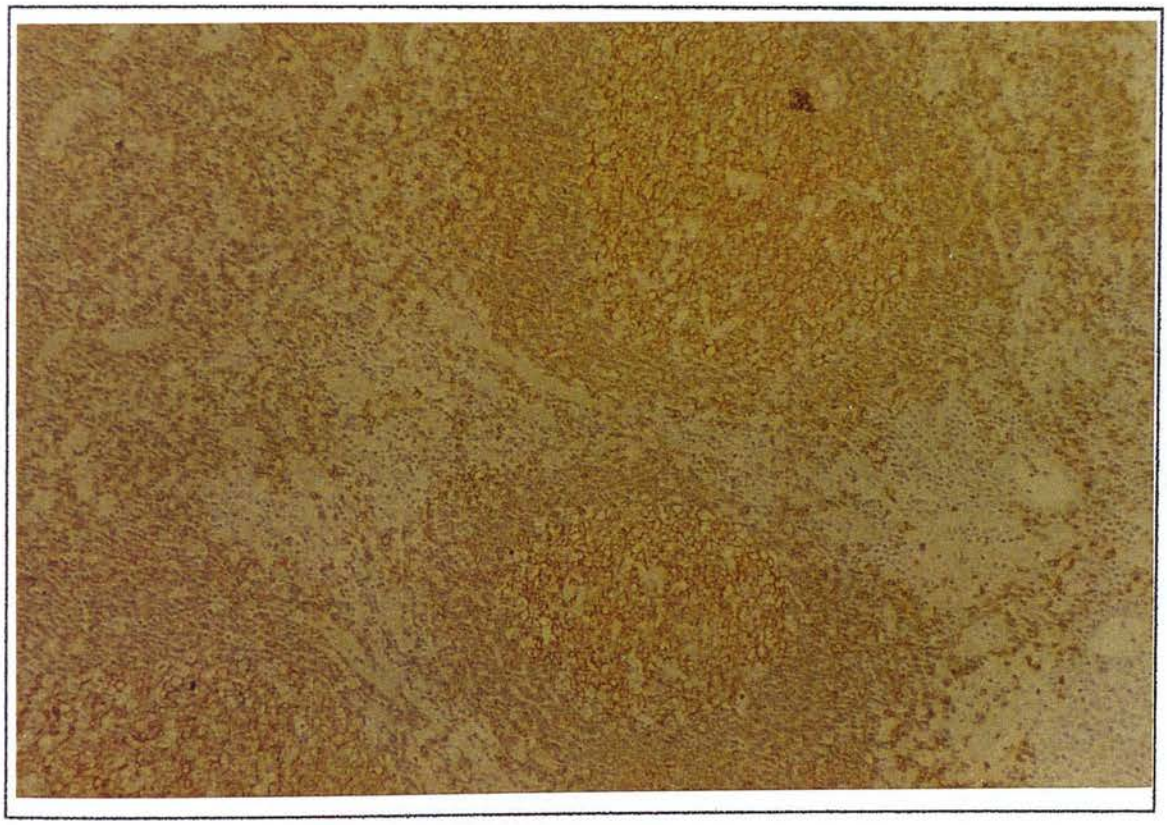
The liver was perhaps the most interesting of the tissues studied. 11 samples were tested, 5 of which came from liver tumours and another 5 which came from more normal parts of liver from these same patients. A summary of staining patterns observed on normal liver and tumour tissue is given in table 4.5.1 below. The 5 liver tumour samples were of various different types of tumours. Unfortunately, no data was obtained for EBU-65 staining as no EBU-65 mAb was available at the time of this study.

Zonal staining was observed with at least one of the three mAb tested in all normal tissues studied. In all cases zone one is stained most intensely. This is the area of the liver closest to the hepatic artery. It seemed very unusual to find increased levels of the products of a sialyltransferase enzyme in this location as most hepatic transferases are found in zone 3 which is further away from the artery and has lower oxygen levels. In all cases studied, CDw75 expression was markedly disrupted in tumour tissue when compared with normal tissue. Plates 4.5.1, 4.5.2 and 4.5.3(a) show examples of the normal staining pattern observed on normal liver with HH2 and EBU-141. In all cases, there is strong canalicular staining which is more concentrated in zone 1. If we look at plate 4.5.3(b) we see an example of EBU-141 binding to tumour tissue. Most cells are unstained, with occasional positive cells being found at the edges of the tumour. This looks like an immature attempt at zonal focality. In most cases, there is little or no binding at all of HH2 and EBU-141 to tumour tissue sections. Plate 4.5.4 shows an unusual example of zonal cytoplasmic staining of a normal liver by OKB4. In most sections studied, OKB4 staining was very weak or non-existent.

Photographic Plate 4.2.1

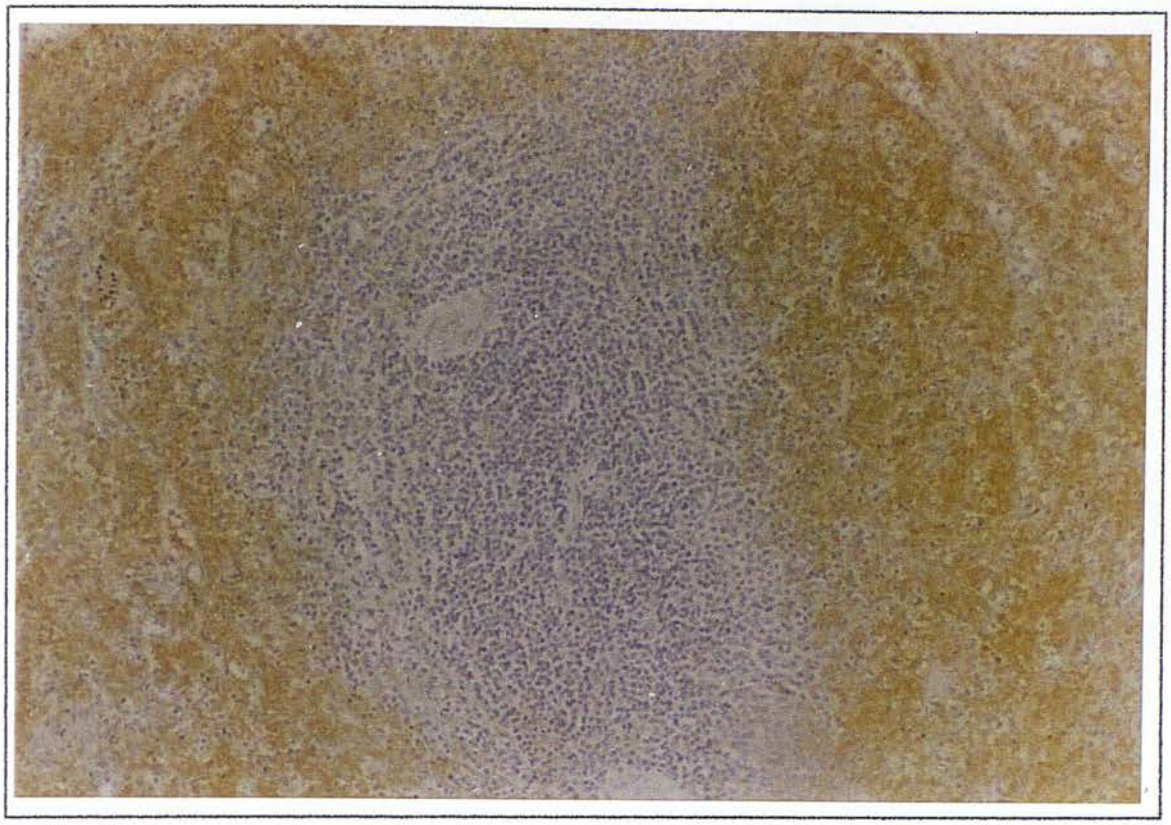


(a) HH2 staining of lymph node 8180/69. B-cell follicles only (x 10 magnification).

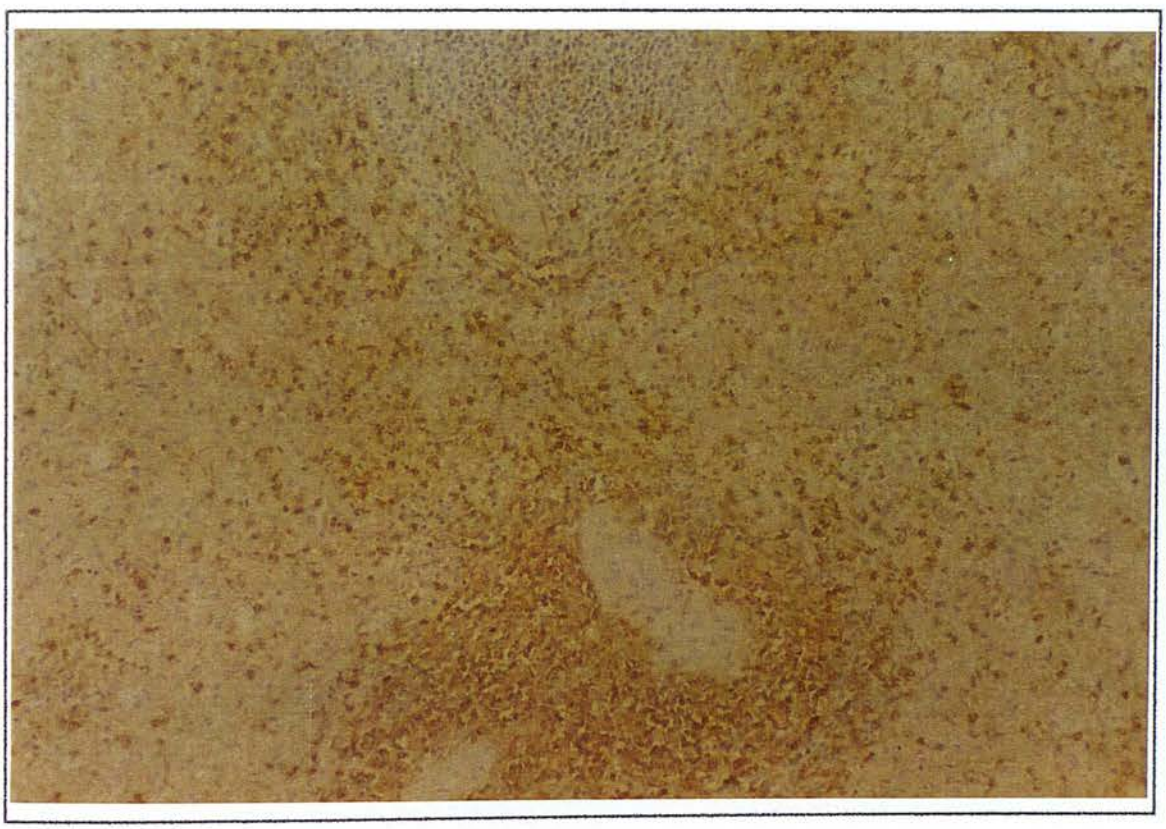


(b) CD19 staining of lymph node 8180/69. Follicles and mantle zones (x 10 magnification).

Photographic Plate 4.3.1

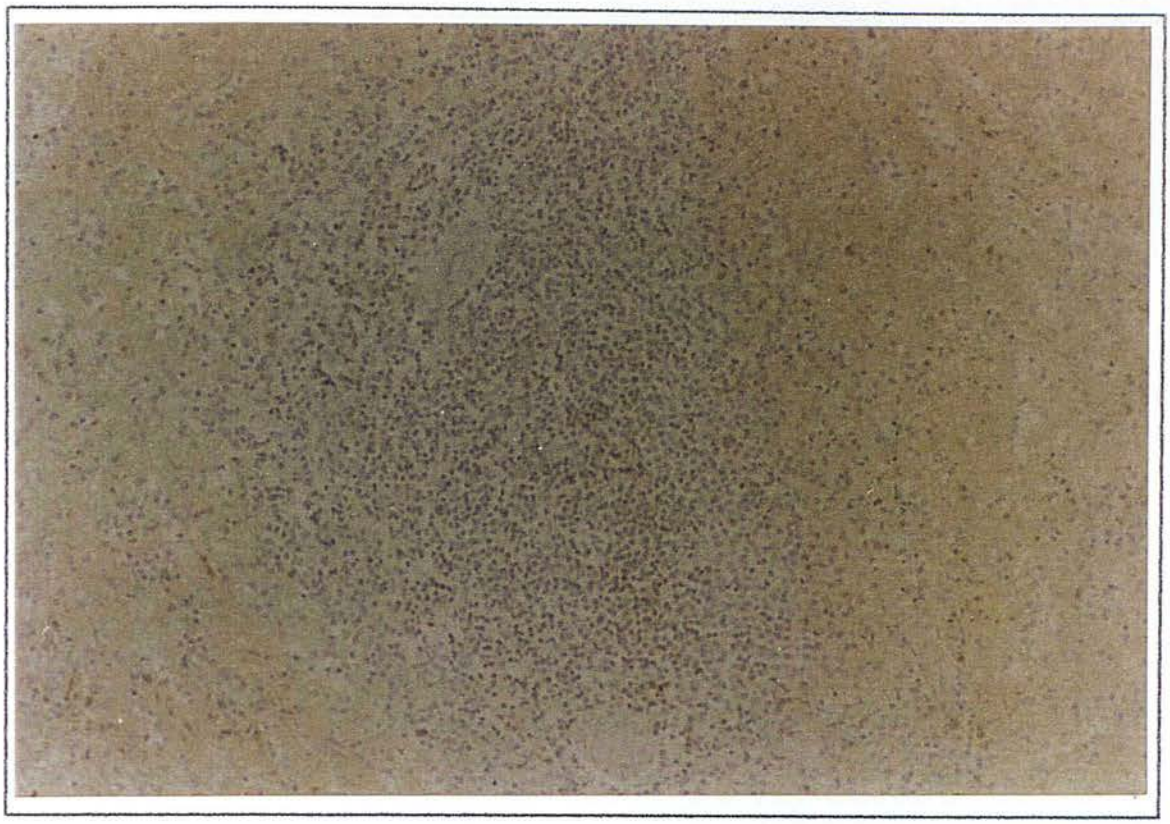


(a) OKB4 staining of spleen 8064/87 (x 10 magnification).

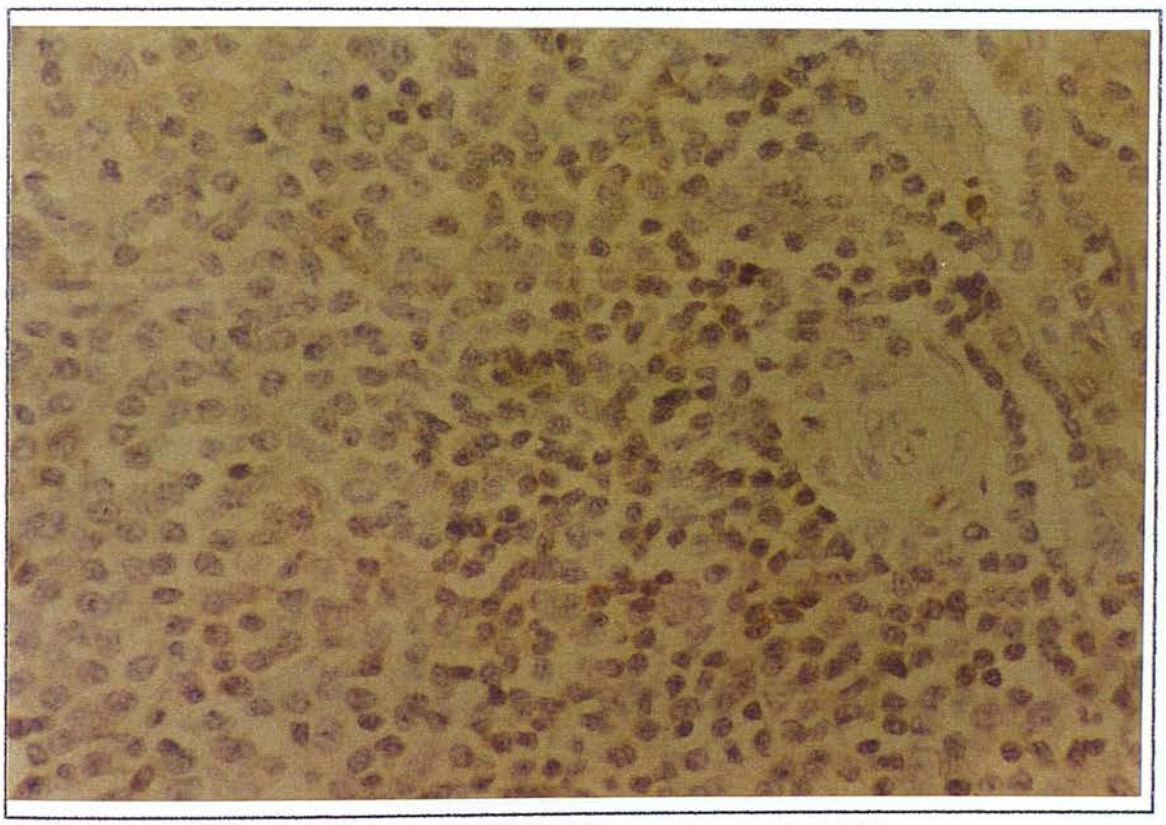


(b) CD3 staining of spleen 8064/87 (x 10 magnification).

Photographic Plate 4.3.2

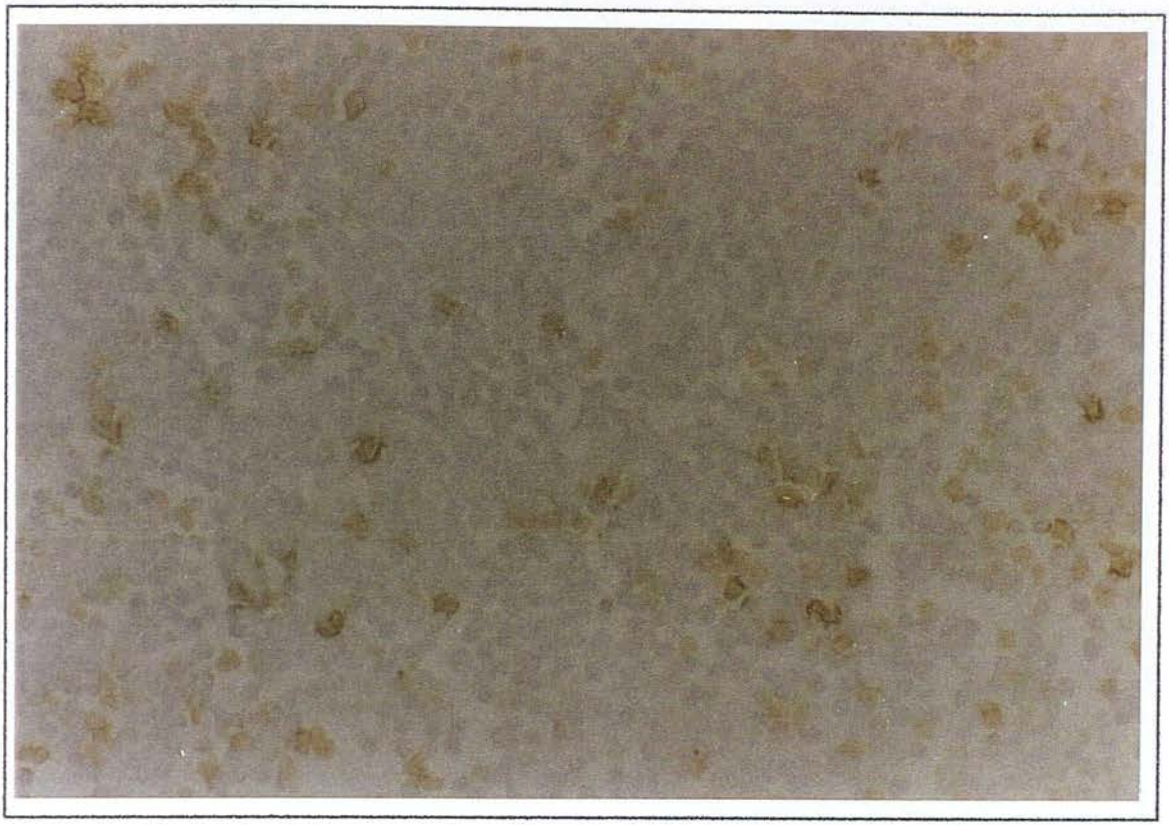


(a) EBU-141 staining of spleen 8064/87 (x 10 magnification).

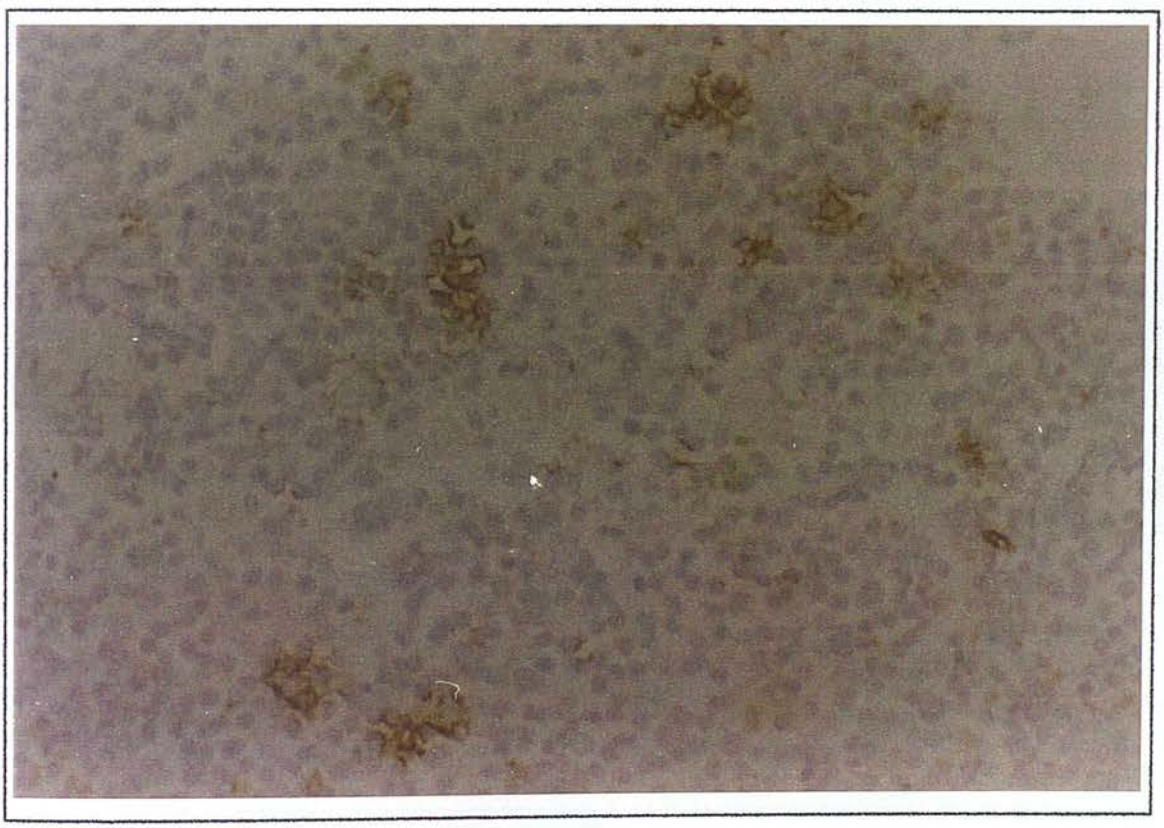


(b) EBU-141 staining of spleen 8064/87 (x 40 magnification).

Photographic Plate 4.4.1

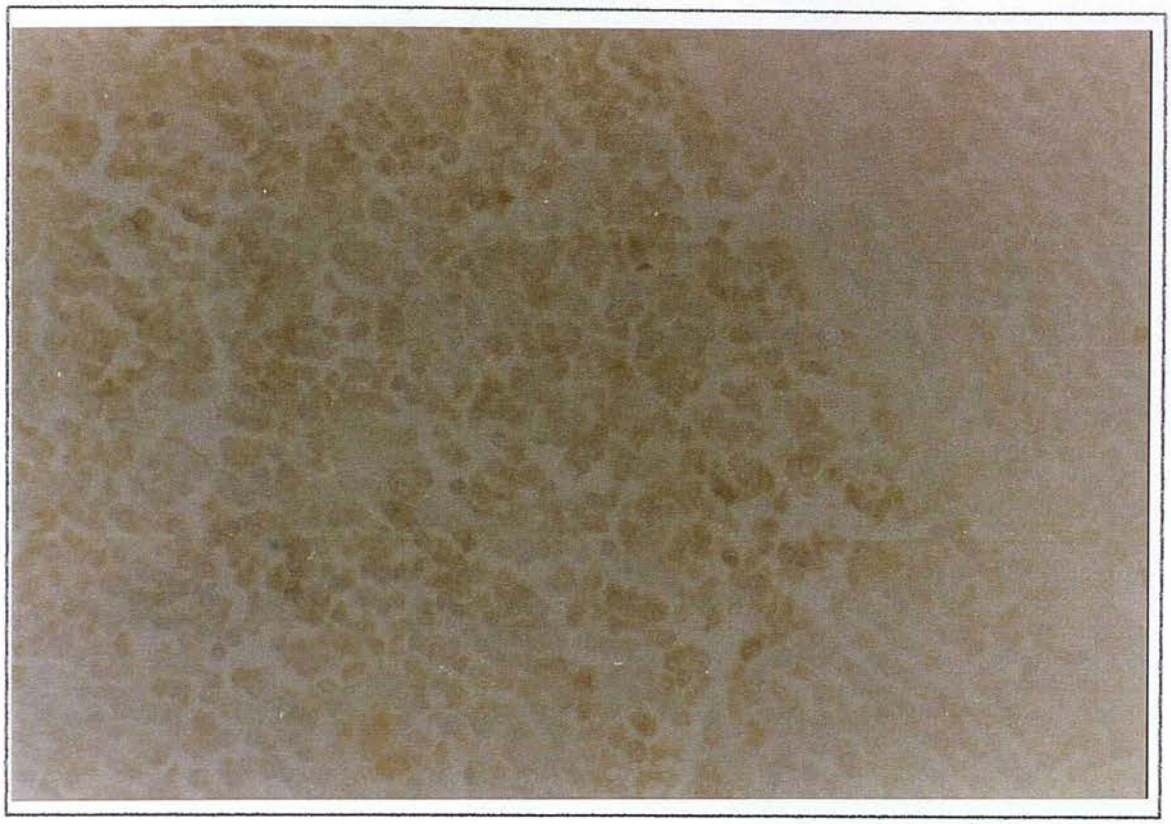


(a) HH2 staining of thymus RL91-582 (x 40 magnification).

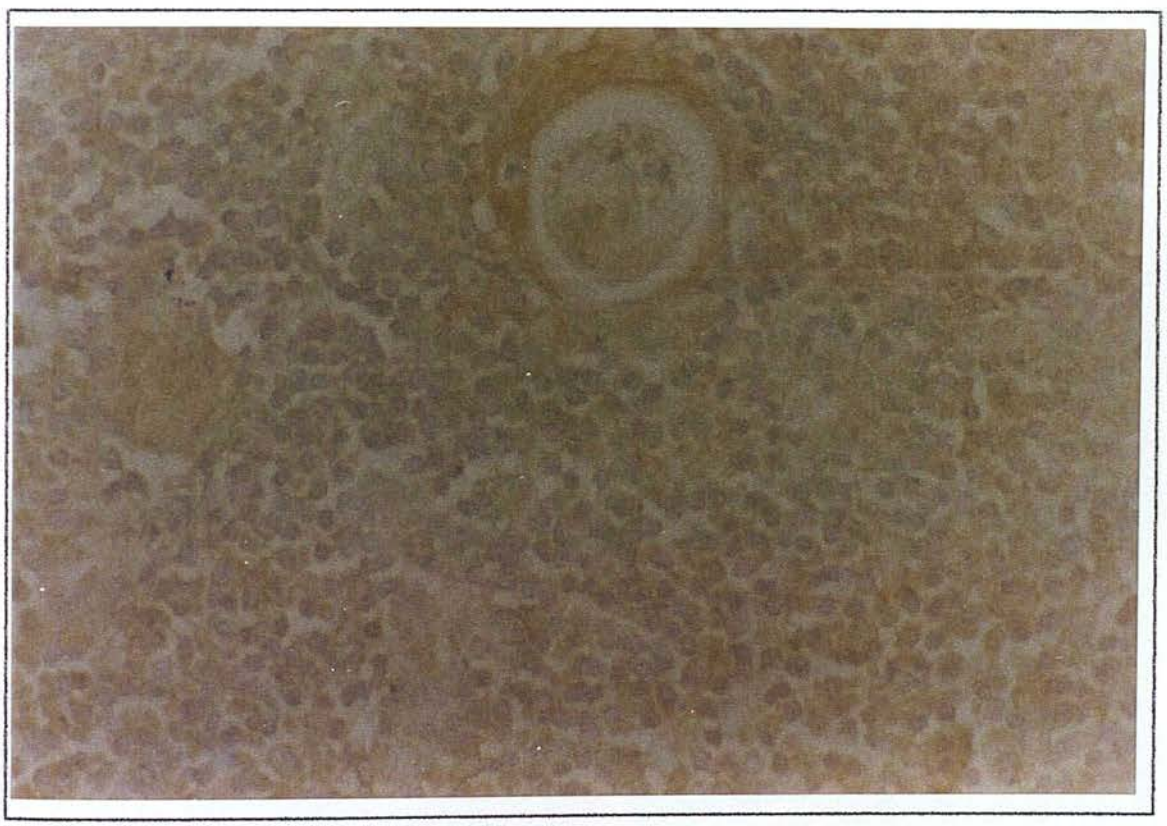


(b) CD19 staining of thymus RL91-582 (x 40 magnification).

Photographic Plate 4.4.2



(a) OKB4 staining of thymus RL91-582 (x 40 magnification).



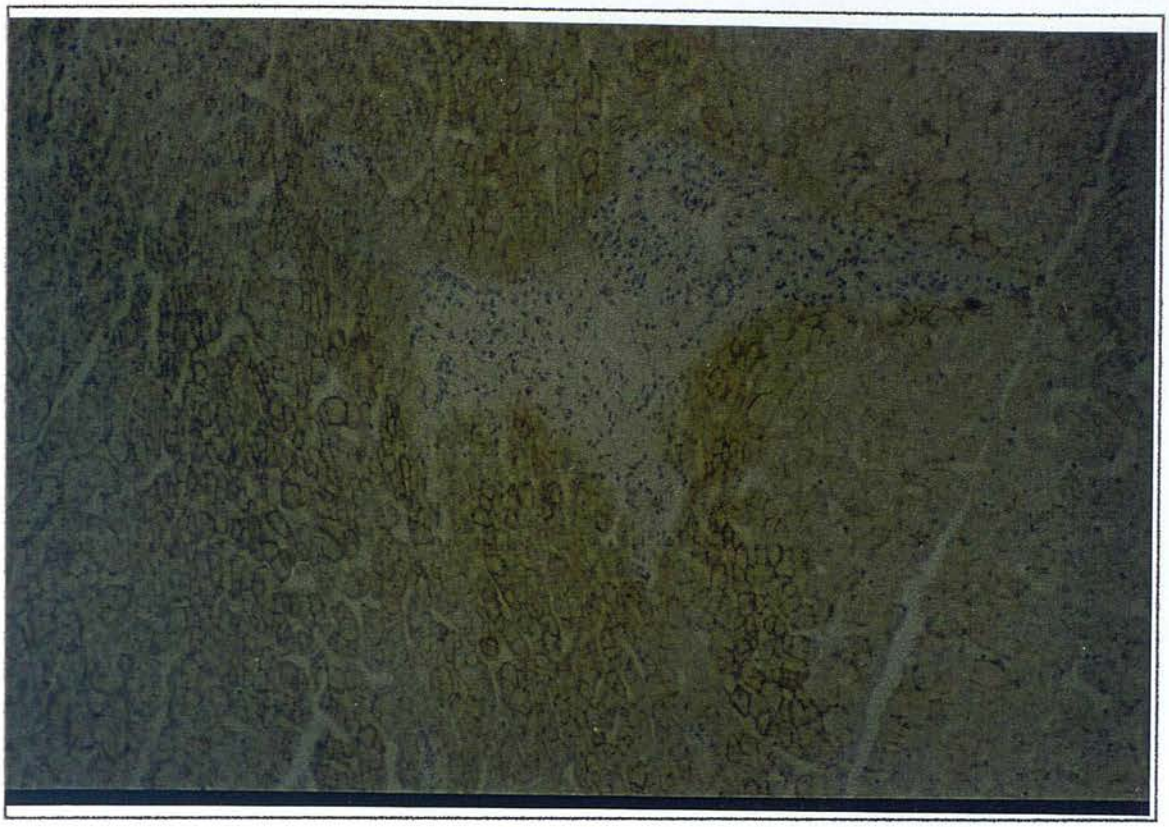
(b) EBU-141 staining of thymus RL91-582 (x 40 magnification).

Photographic Plate 4.5.1

(a) HH2 staining of liver 1 (normal) (x 40 magnification).

(b) HH2 staining of liver 3 (normal) (x 10 magnification).

Photographic Plate 4.5.2

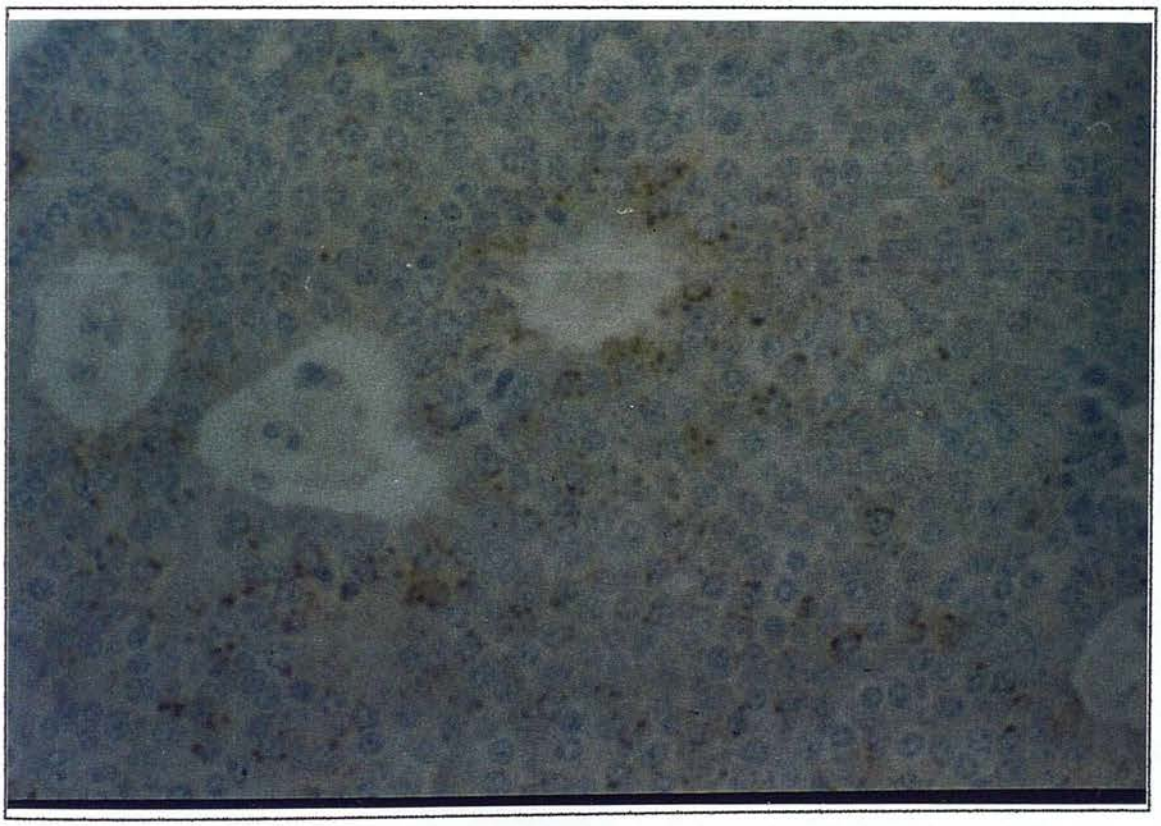


(a) HH2 staining of liver 4 (normal) (x 10 magnification).

(b) EBU-141 staining of liver 4 (normal) (x 10 magnification).

Photographic Plate 4.5.3

(a) EBU-141 staining of liver 3 (normal) (x 10 magnification).



(b) EBU-141 staining of liver 3 (tumour) (x 20 magnification).

Photographic Plate 4.5.4

(a) OKB4 staining of liver 6 (normal) (x 10 magnification).

Table 4.5.1 Results of CDw75 staining on liver.

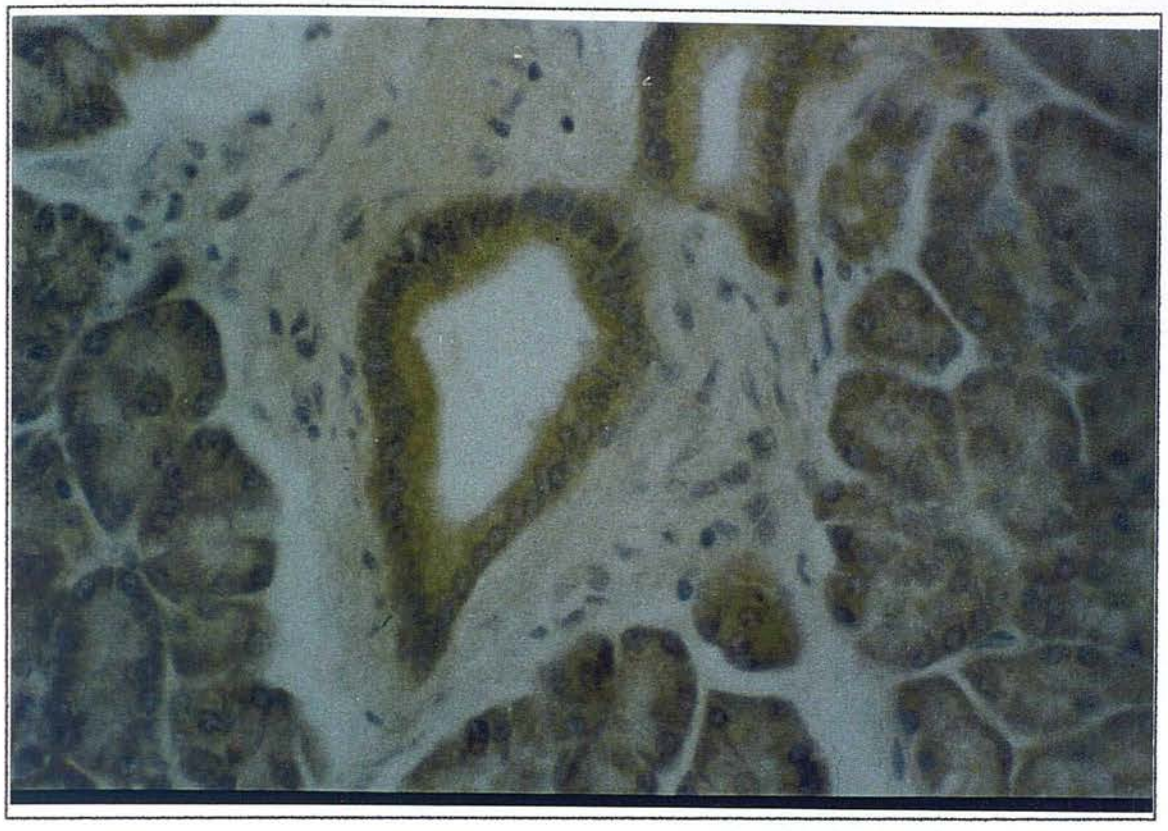
Sample	HH2	OKB4	EBU-141
1	strong canalicular staining esp. in zone 1	negative	strong canalicular staining esp. in zone 1
1(t)	negative	background stroma and cells at edges of malignant tumours and canaliculae	negative
2	zonal staining of canaliculae	uniform weak cytoplasmic staining	zonal staining of canaliculae
2(t)	strong staining at edges of tumour only	negative	strong staining at edges of tumour only
3	strong canalicular staining esp. in zone 1	weak cytoplasmic staining only	zonal staining of canaliculae
3(t)	mostly negative, few stained cells at edges of tumour	negative	as HH2, immature attempt at zonal focality?
4	strong canalicular staining esp. in zone 1	weak cytoplasmic staining, stronger on bile ducts	strong canalicular staining esp. in zone 1
4(t)	stains duct-forming cells	stains tumour cells	stains duct-forming cells
5	zonal staining of canaliculae	negative	zonal staining of canaliculae
5(t)	few small cells with cytoplasmic staining, membranes of large multi-nucleate cells	few small cells with cytoplasmic staining, membranes of large multi-nucleate cells	few small cells with cytoplasmic staining, membranes of large multi-nucleate cells
6	zonal staining of canaliculae	Pale cytoplasmic, much stronger in zone 1	zonal staining of canaliculae

It is also curious that the canaliculae of the liver are stained with CDw75. This was previously unreported. LN-1, EBU-141 and EBU-65 mAb have been reported to bind to hepatocytes (Epstein, A.L., Marder, R.J., Winter, J.N. and Fox, R.I., 1984; Gramatzki, M., Burger, R., Kraus, J., Lauer, U., Rohwer, P., Eger, G., Kalden, J.R. and Henschke, F., 1991), but the pattern of expression in tissue sections is not described. There are no reports of HH2 binding on liver cells.

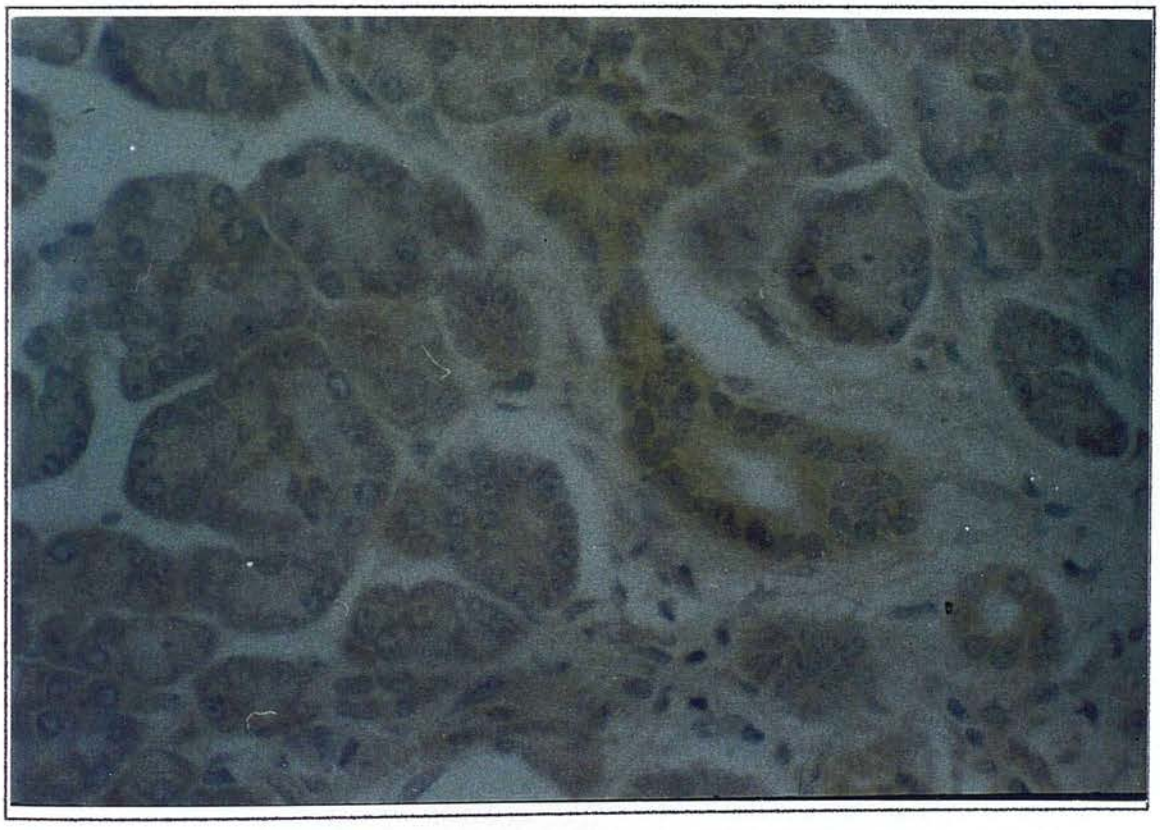
4.6 CDw75 Expression on Pancreas.

Nine samples of pancreas tissue were studied from normal and cancerous parts of pancreas from five patients. Sections were stained with four CDw75 mAb - HH2, OKB4, EBU-141 and EBU-65. Some sections were also stained with SNA, using a biotinylated conjugate of this lectin. Results of pancreatic staining by CDw75 are summarised in table 4.6.1. In the normal samples studied, CDw75 expression was found to be mainly confined to the cytoplasm of ductal cells and acini. Examples of the normal CDw75 expression pattern can be seen in photographic plates 4.6.1 and 4.6.2(a), and of HH2 staining of tumour tissue in plate 4.6.2(b).

Photographic Plate 4.6.1



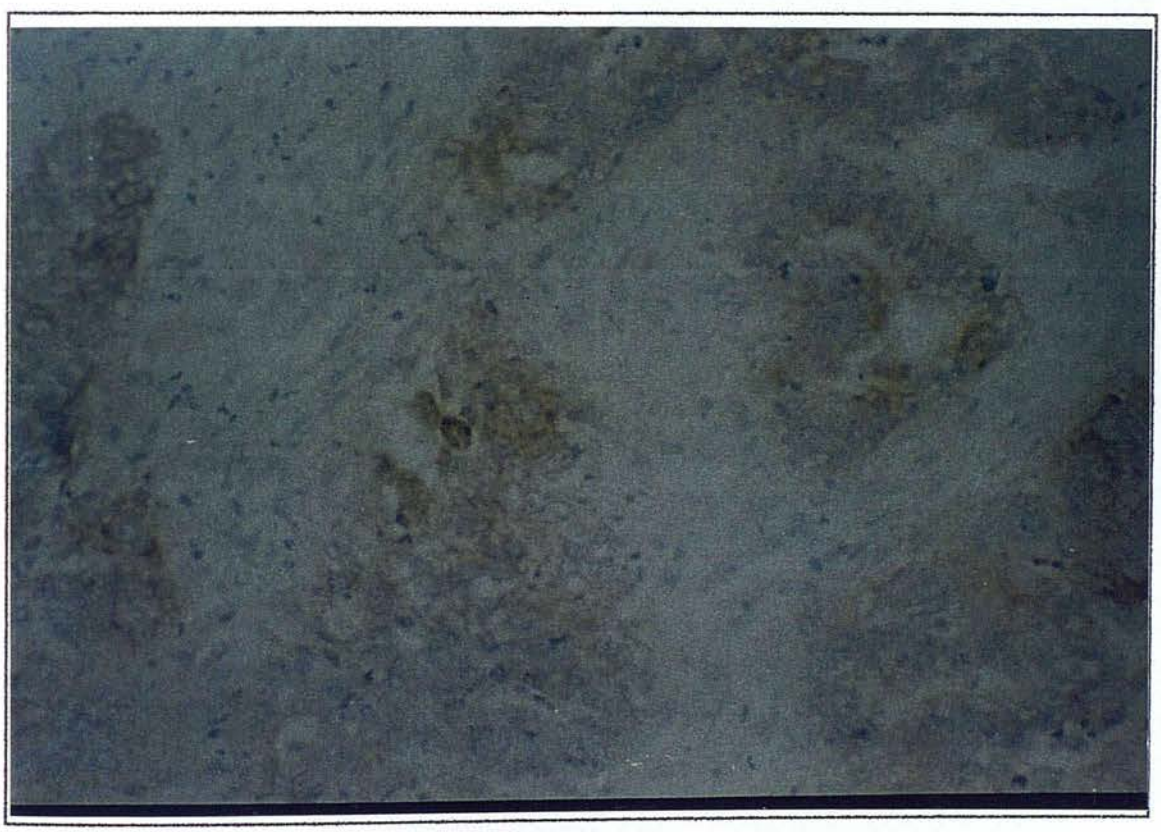
(a) OKB4 staining of pancreas 2 (normal) (x 40 magnification).



(b) EBU-141 staining of pancreas 2 (normal) (x 40 magnification).

Photographic Plate 4.6.2

(a) EBU-65 staining of pancreas 2 (normal) (x 40 magnification).



(b) HH2 staining of pancreas 2 (tumour) (x 10 magnification).

HH2 was not found to strongly stain any of the normal tissue samples studied, but as can be seen in the photograph, bound to some cells in the tumour-damaged ducts. SNA staining was variable, but mainly restricted to the ductal cells of normal pancreas tissue. Islet cells were stained in one sample studied. LN-1 has been reported to bind to ductal cells in many organs, including the pancreas (Epstein, A.L., Marder, R.J., Winter, J.N. and Fox, R.I., 1984), but no staining of pancreatic ducts or acini is reported for EBU-141 or EBU-65 (Gramatzki, M., Burger, R., Kraus, J., Lauer, U., Rohwer, P., Eger, G., Kalden, J.R. and Henschke, F., 1991).

Table 4.6.1 Results of CDw75 staining on pancreas.

Sample	HH2	OKB4	EBU-141	EBU-65	SNA
1	negative	cytoplasmic staining of ductal cells	cytoplasmic staining of ducts, acini and islet cells	cytoplasmic staining of ducts, acini and islet cells	negative
1(t)	negative	weak cytoplasmic staining of tumour	weak cytoplasmic staining of tumour	weak cytoplasmic staining of tumour	N.D.
2	weak staining of ducts & acini	strong cytoplasmic staining of ducts & acini	cytoplasmic staining of ducts & acini	cytoplasmic staining of ducts & acini, (stronger on ducts)	N.D.
2(t)	focal staining of damaged ducts	negative	patchy staining of membranes and cytoplasm	patchy staining of membranes and cytoplasm	N.D.
3	negative	cytoplasmic staining of ducts & acini	cytoplasmic staining of ducts, acini weakly stained	cytoplasmic staining of ducts, acini weakly stained	cytoplasmic staining of ducts only
3(t)	negative	N.D.	cytoplasm of tumour cells weakly stained	weak cytoplasmic staining of tumour cells	cytoplasm of tumour cells weakly stained
4	negative	cytoplasmic staining of ducts & acini	cytoplasmic staining of ducts & acini	cytoplasmic staining of ducts & acini (weak)	Strong staining of islet cells only
4(t)	negative	strong, patchy cytoplasmic and membrane staining	cytoplasmic staining of tumour cells	strong but patchy cytoplasmic staining	negative
5	negative	cytoplasmic staining of ducts & acini	cytoplasmic staining of ducts & acini	cytoplasmic staining of ducts, acini weakly stained	weak cytoplasmic staining of ducts & acini

In all cases studied, CDw75 expression was very disrupted in the tumour samples when compared with the normal samples. Staining of tumour cells was always much weaker and patchier than on normal cells. It appears that CDw75 is not synthesised

properly in pancreatic tumour cells. It is either not expressed at all or is improperly glycosylated. In some cases its expression is increased in focal areas.

4.7 CDw75 Expression on Small Intestine and Colon.

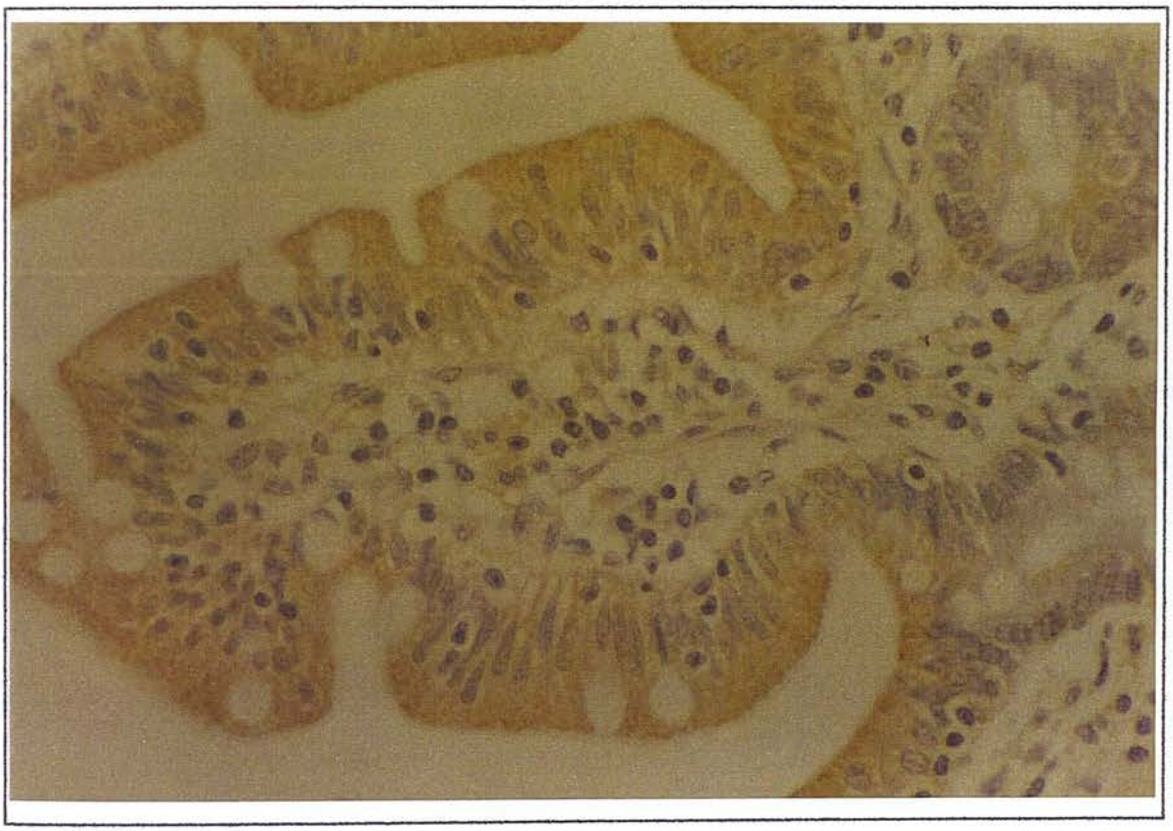
Two samples of small intestine tissue were obtained from a patient with a fibroma (4013/88) and from another patient with a leiomyoma. OKB4 was the only CDw75 mAb to stain sections of either of these samples. The results of OKB4 staining of tissue sample 4013/88 are shown in plate 4.7.1. OKB4 stained the epithelial cells of this section only.

Ten samples of colon tissue from a variety of sources were studied (some normal, some diseased, and some cancerous). Results from CDw75 staining of these samples were mainly negative except in two abnormal samples. These came from a patient with a rectal polyp(4), and from another patient with acute ulcerative colitis induced by a benign gastric tumour(3). In both cases, the endothelial cells of the villi were stained by all four CDw75 mAb tested. Staining was most intense at the tips of the villi. This can be seen in photographic plates 4.7.2 - 4.7.4. A summary of results obtained is given in table 4.7.1.

LN-1 staining of the superficial epithelium has been reported, as well as staining of epithelium-derived tumour cells of the colon (Epstein, A.L., Marder, R.J., Winter, J.N. and Fox, R.I., 1984). EBU-141 and EBU-65 have also been reported to stain intestinal mucosa and endothelial cells (Gramatzki, M., Burger, R., Kraus, J., Lauer, U., Rohwer, P., Eger, G., Kalden, J.R. and Henschke, F., 1991). It is not clear from these reports if CDw75 was found to be expressed in all intestine samples tested. Certainly from my own results, it would appear that expression is only induced in abnormal intestinal cells.

Photographic Plate 4.7.1

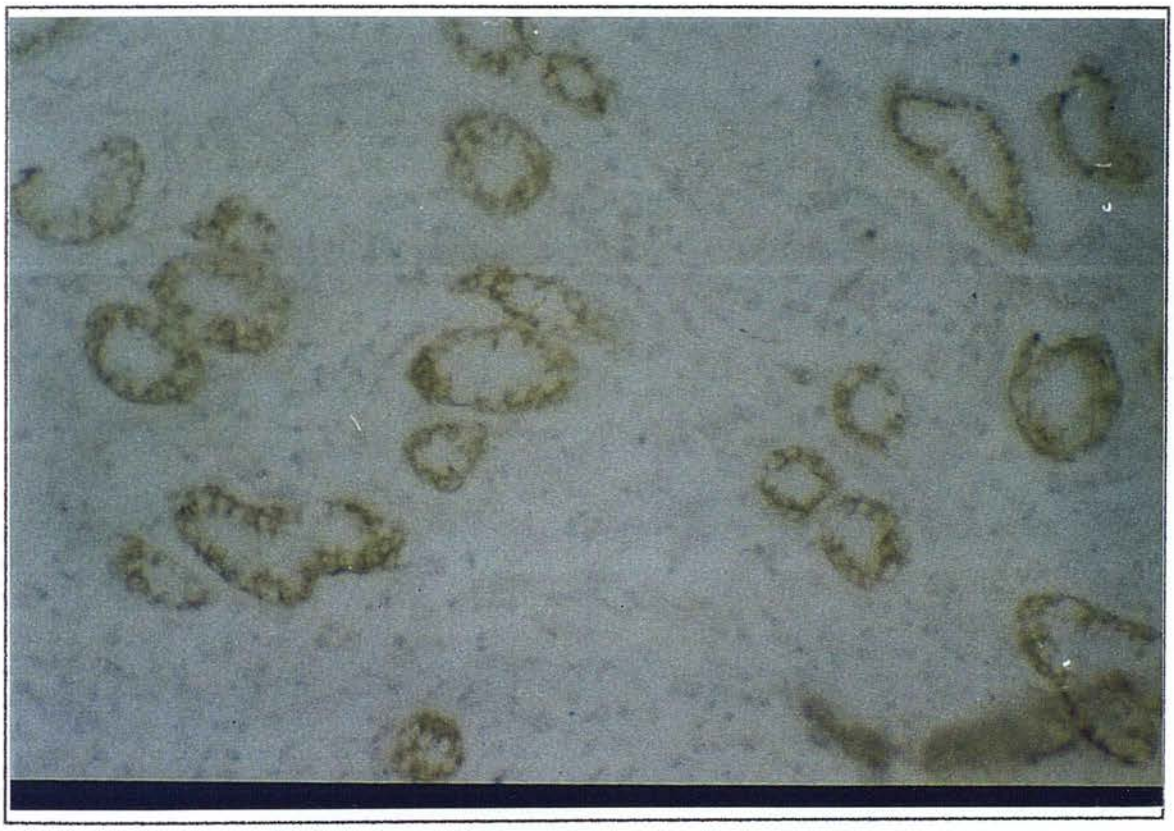
(a) OKB4 staining of small intestine 4013/88 (x 10 magnification).



(b) OKB4 staining of small intestine 4013/88 (x 40 magnification).

Photographic Plate 4.7.2

(a) EBU-65 staining of colon 3 (x 10 magnification).



(b) HH2 staining of colon 3 (x 10 magnification).

Photographic Plate 4.7.3

(a) OKB4 staining of colon 3 (x 10 magnification).

(b) EBU-65 staining of colon 3 (x 10 magnification).

Photographic Plate 4.7.4

(a) HH2 staining of colon 4 (x 40 magnification).

Table 4.7.1 Results of CDw75 staining on colon and small intestine.

Sample	HH2	OKB4	EBU-141	EBU-65
1 normal	negative	negative	negative	negative
2 rectal ca	negative	negative	negative	negative
3 gastric ca	positive in glands	positive in glands	positive in glands	positive in glands
4 rectal polyp	cytoplasmic staining at tips of villi	cytoplasmic staining at tips of villi	cytoplasmic staining at tips of villi	cytoplasmic staining at tips of villi
5 colorectal ca	negative	negative	negative	negative
6 caecal ca	negative	negative	negative	negative
7 normal	negative	negative	negative	negative
8 normal	negative	negative	negative	negative
9 anaplastic colloid ca	negative	negative	negative	negative
10 normal	negative	negative	negative	negative
S.I. 4013/88	negative	cytoplasmic staining of villi enterocytes	negative	negative
S.I. 23631/88	negative	negative	negative	negative

4.8 CDw75 Expression on Breast.

Breast tissue was very difficult to study as samples often consisted of mainly fatty tissue with very few distinguishable cells. Of the ten samples studied, four produced results which could be analysed. All four of these samples were taken from breast tumours. "Normal" samples taken from the same patients were in too poor a condition for useful study.

Where staining was found, it was mainly on ductal cells, and clear examples of this ductal staining on breast tissue are demonstrated in plates 4.8.1 and 4.8.2. In one sample (4.8.3), weaker cytoplasmic staining of all tumour cells was found, with comparatively stronger staining on the ducts. Results of breast tissue staining are summarised in table 4.8.1.

Table 4.8.1 Results of CDw75 staining on breast.

Sample	HH2	OKB4	EBU-141	EBU-65
3	ducts stained only	ducts stained only	ducts stained only	ducts stained only
5	tumour negative, normal cells and ducts only stained	tumour cells and ducts stained	tumour cells and ducts stained	tumour cells and ducts stained
7	negative	negative	negative	negative
9	negative	negative	negative	ducts and lobules stained

Staining of ducts and lobules of the breast with LN-1 has been reported (Epstein, A.L., Marder, R.J., Winter, J.N. and Fox, R.I., 1984). However, EBU-141 and EBU-65 were not found to stain breast tissue (Gramatzki, M., Burger, R., Kraus, J., Lauer, U., Rohwer, P., Eger, G., Kalden, J.R. and Henschke, F., 1991). Photographs on plate 4.8.2 show clear examples of EBU-141 and EBU-65 staining of breast tumour which contradicts these findings. Perhaps expression is limited only to tumour tissue which may not have been tested by Gramatzki *et al.* It is unfortunate that no useful samples of normal tissue were available for comparative staining in this study.

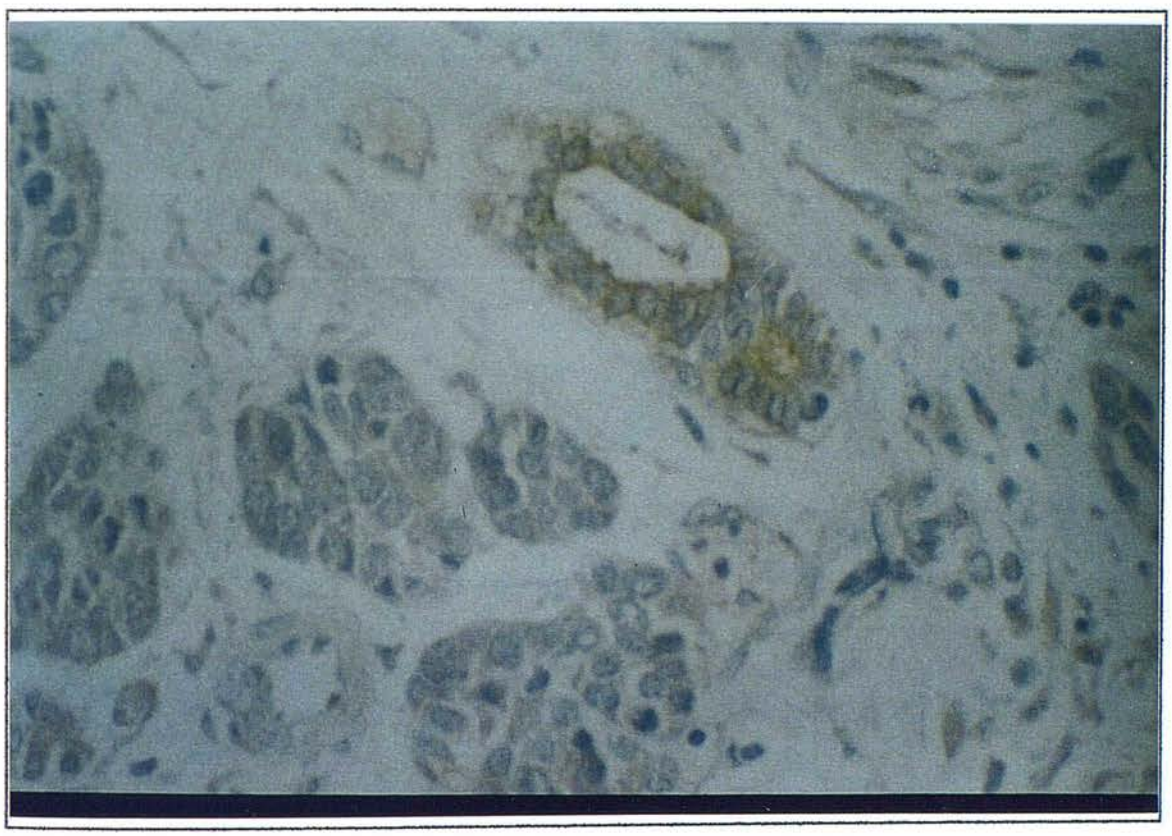
4.9 CDw75 Expression on Lung.

Only one sample of normal lung tissue was stained (block 23104/90). HH2 appeared to stain the epithelium of bronchioles. OKB4 staining of the epithelium was stronger than that of HH2, and stained with even greater intensity after neuraminidase treatment of the section. These results are illustrated in photographic plate 4.9.1. No staining of this sample was observed with EBU-65 and only very weak epithelial staining was achieved with EBU-141.

LN-1 is not reported to stain lung tissue, but staining of the ciliated epithelial cells of the bronchus is reported (Epstein, A.L., Marder, R.J., Winter, J.N. and Fox, R.I., 1984). There are no reports in the literature of staining of lung tissue with any of the other CDw75 mAbs.

Photographic Plate 4.8.1

(a) HH2 staining of breast 3 (x 40 magnification).



(b) OKB4 staining of breast 3 (x 40 magnification).

Photographic Plate 4.8.2

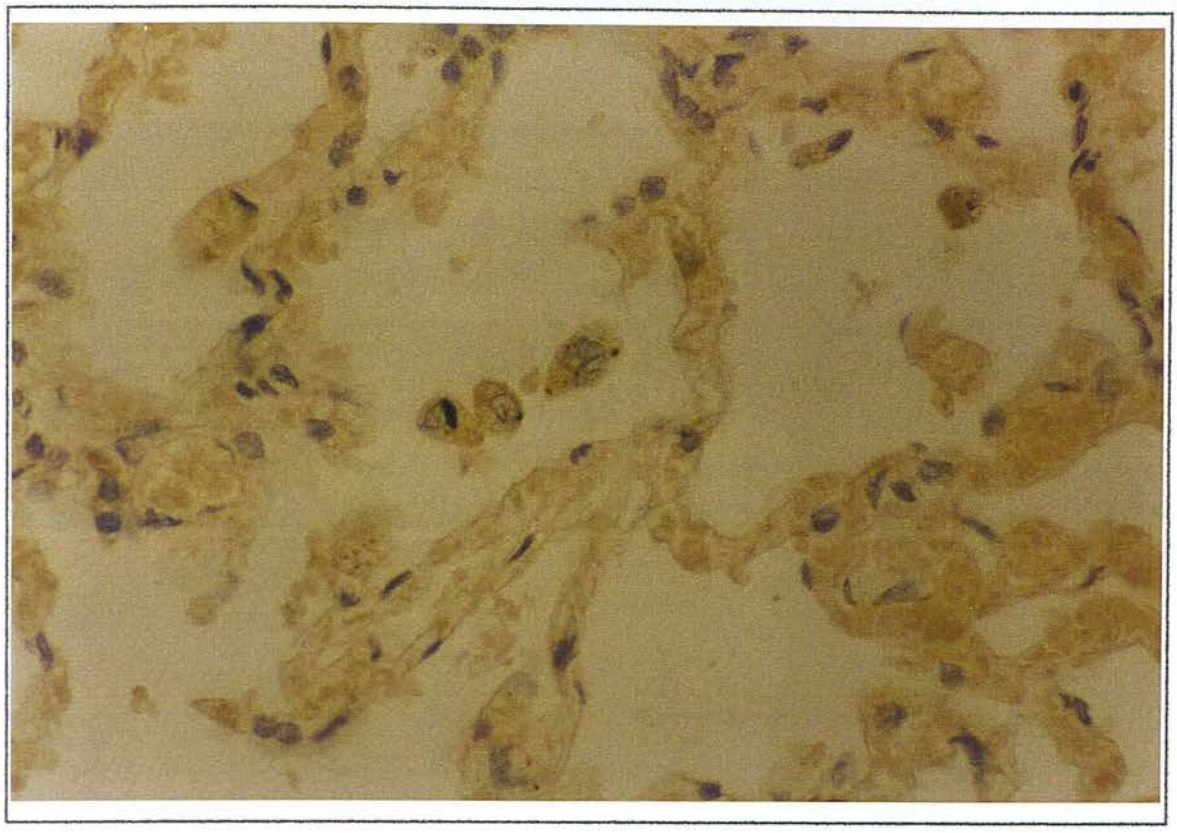
(a) EBU-141 staining of breast 3 (x 40 magnification).

(b) EBU-65 staining of breast 9 (x 40 magnification).

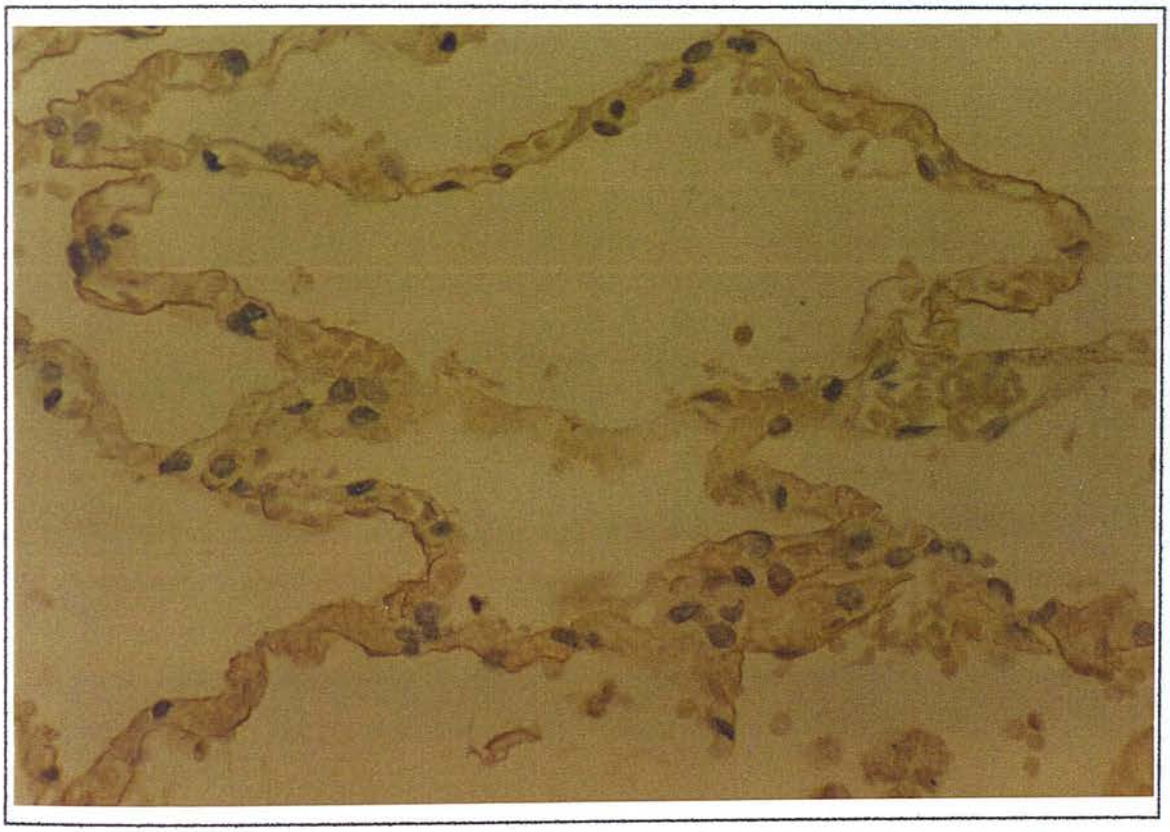
Photographic Plate 4.8.3

(a) HH2 staining of breast 5 (x 40 magnification).

Photographic Plate 4.9.1



(a) OKB4 staining of lung 2310/90 (x 40 magnification).



(b) OKB4 staining of lung 2310 4/90 after neuraminidase treatment (x 40 magnification).

4.10 Discussion.

It is generally accepted that carbohydrates are of major importance in all areas of embryogenesis, cell-differentiation and malignancy. For example, vital cell functions such as growth can be completely disrupted when oligosaccharide chains are disturbed by the action of anti-carbohydrate mAbs causing autophosphorylation of epidermal growth factor receptors(Feizi, T., 1990). More specifically, increased levels of cell-surface sialylation have been linked with cell transformation and increased metastasising potential(Yogeeswaran, G. and Salk, P.L., 1981; Hakomori, S., Patterson, C.M., Nudelman, E. and Sekiguchi, K., 1983; Smets, L.A. and Van Beek, W.P., 1984; Dennis, J.W., 1986; Humphries, M.J., Matsumoto, K., White, S.L. and Olden, K., 1986; Cohen, A.M., Allalouf, D., Djaldetti, M., Weigl, K., Lehrer, N. and Levinsky, H., 1989; Dall'Olio, F., Malagolini, N., di Stefano, G., Minni, F., Marrano, D. and Serafini-Cessi, F., 1989; Francina, A., Gateau-Roesch, O., Couprie, N., Leculier, C., Col, J.F., Archimbaud, E., Campos, L., Louisot, P. and Richard, M., 1989; Springer, G.F., 1989; Bresalier, R.S., Rockwell, R.W., Dahiya, R., Duh, Q.Y. and Kim, Y.S., 1990; Easton, E.W., Bolscher, J.G.M. and van den Eijnden, D.H., 1991; Sasaki, H., Momoi, T., Yamanaka, C., Yorifuji, T., Kaji, M. and Mikawa, H., 1991; Harvey, B.E., Toth, C.A., Wagner, H.E., Steele, G.D.J. and Thomas, P., 1992; Vandamme, V., Cazlaris, H., Le Marer, N., Laudet, V., Lagrou, C., Verbert, A. and Delannoy, P., 1992). It has been suggested that increased sialylation of cell-surface glycoconjugates, whether glycoprotein or glycolipid, may contribute to the metastatic behaviour of tumour cells through (a) an increased adhesiveness leading larger tumour cell emboli or to an increased capacity to adhere to vascular endothelium at secondary sites of implantation; or (b) an increased capacity to aggregate blood platelets; or (c) a decrease in the susceptibility of the cells to destruction by host immune mechanisms(Yogeeswaran, G. and Salk, P.L., 1981).

Although the significance of the findings of this study is somewhat limited due to the quality and quantity of tissue samples available, several differences were observed in the distribution of CDw75 in normal and tumour tissues which correlate with previously observed differences in enzyme activities observed in these tissues.

Samples of lymphoid tissue were very scarce, but on those studied, CDw75 expression was limited to the B-cell areas. However, in tonsil and lymph node mAbs did not stain all B-cells, as staining tended to be restricted to follicular areas. In spleen, likewise staining was restricted to the B-cells of the white pulp, and in thymus there

was no significant staining. There were not enough samples available to make any comparisons between sections of normal and cancerous lymphoid tissues.

CDw75 expression on normal liver was almost always restricted to the canaliculae, whereas in liver tumour samples taken from the same patients, CDw75 staining was totally disrupted. It is not clear whether this is due to a morphological change in the cells of the tumour causing destruction of the network of canaliculae, or whether the enzyme β-galactoside-α-2,6-sialyltransferase is downregulated in liver cancer. In two samples, only the cells at the edges of the tumour were CDw75 positive, almost as if the cells were attempting to form a canaliculae-like structure. It is known that there are receptors for serum galactose terminated glycoproteins in the liver(Cook, J., Hou, E., Hou, Y., Cairo, A. and Doyle, D., 1983), and it is possible that captured proteins are then sialylated here. However, CDw75 staining of canaliculae has not previously been reported. A further investigation of CDw75 expression on transformed hepatocytes coupled with an assay of sialyltransferase activities and mRNA levels would be necessary in order to draw any conclusions from these findings. However it is possible to speculate that although in many types of cancer there are increased levels of α-2,6-sialyltransferase, in liver cancer this may not be the case. The reduced expression of CDw75 may reflect the upregulation of a different glycosyltransferase, possibly causing increased branching of the carbohydrate moiety and differential terminal glycosylation. It has been shown that the same core protein can be differently glycosylated in different tissues, and that yet more differences can be induced upon malignant transformation(Taylor-Papadimitriou, J., 1991). This may also be the case in pancreatic cancer. The differences in CDw75 staining between normal and malignant areas of the same pancreas were not as marked as in the liver samples studied. However as with the liver samples, CDw75 binding was reduced in the pancreatic tumour samples when compared to non-tumour samples.

In the colon samples studied, CDw75 was only expressed on malignant cells. This may reflect an increase in α-2,6-sialyltransferase activity in transformed colon cells. Others have reported increased SNA binding to the surface of some metastatic colon cell lines when compared to parental lines(Bresalier, R.S., Rockwell, R.W., Dahiya, R., Duh, Q.Y. and Kim, Y.S., 1990) and a corresponding increase in α-2,6-sialyltransferase activity in these transformed cells. Others have monitored α-2,6-sialyltransferase activities in normal human colonic epithelium and in colorectal carcinomas and have also found increased levels of activity in the tumour cells(Dall'Olio, F., Malagolini, N., di Stefano, G., Minni, F., Marrano, D. and

Serafini-Cessi, F., 1989). This was reflected in an increased level of α-2,6-sialylated cell-surface glycoproteins and glycolipids. These sialylated glycolipids have also been detected in corresponding foetal tissues, and therefore they may perhaps be described as oncofoetal antigens which arise due to the activation of a foetal enzyme during neoplastic transformation(Hakomori, S., Patterson, C.M., Nudelman, E. and Sekiguchi, K., 1983). Others have shown a direct correlation between α-2,6-sialyltransferase activities in colon cancer cells and their metastatic potential(Harvey, B.E., Toth, C.A., Wagner, H.E., Steele, G.D.J. and Thomas, P., 1992). Many of the samples used in this study did not express significant levels of CDw75. It may be that these samples were from patients with less aggressive tumours which may not have formed metastases. There are also reports of differential fucosylation of glycoproteins in certain colon carcinomas(Stroup, G.B., Anumula, K.R., Kline, T.F. and Caltabiano, M.M., 1990). It is possible that some of the samples used in this study may have been of a similar type, and thus had altered fucosylated structures and no changes in sialylation patterns.

In another recent and more comprehensive study comparing CDw75 expression on human gastric carcinomas, it was found that in the majority of cases HH2 did not stain normal gastric tissue but did stain 47% of tumour samples tested(David, L., Nesland, J.M., Funderud, S. and Sobrinho-Simões, M., 1993). Moreover, it was also found that HH2 expression on these tumours correlated with tumour aggression as measured by metastases of the primary tumour into the lymph nodes. The limited results of my own study and those of this more comprehensive study suggest the possibility that CDw75 is not normally expressed on gatrointestinal tissue, but that expression may be induced upon transformation of these cells into tumour cells.

In breast tissue, the study was limited to four samples taken from breast tumours. It is not known if these were primary or secondary tumours. CDw75 staining was observed in three out of the four samples tested, and expression was limited to ductal cells and tumour cells. As previously mentioned, LN-1 mAb has been shown to stain ducts and lobules of normal breast tissue and to strongly stain cytoplasmic filaments of a breast carcinoma cell line(Epstein, A.L., Marder, R.J., Winter, J.N. and Fox, R.I., 1984). EBU-141 and EBU-65 did not stain normal breast tissue in a previous study(Gramatzki, M., Burger, R., Kraus, J., Lauer, U., Rohwer, P., Eger, G., Kalden, J.R. and Henschke, F., 1991), but photographs on plate 4.8.2 clearly show that they do stain breast carcinoma cells. It is therefore possible that α-2,6-sialyltransferase is upregulated in some forms of breast cancer. Indeed in one study,

LN-1 was shown to stain the entire ductal epithelium of 7 different fibroadenoma samples, and the ductal remnants of samples of cysto sarcomas phyllodes samples(Mechtersheimer, G., Krüger, K.H., Born, I.A. and Möller, P., 1990). Other groups have shown a correlation between blood group antigen A expression and aggressive breast tumour behaviour, but also acknowledge that some other groups have shown that colorectal and breast cancers express α-2,6-sialylated forms of this antigen(Springer, G.F., 1989).

Only one sample of normal lung tissue was available, and this produced epithelial staining with all CDw75 mAbs tested. There are reports of LN-1 staining of the ciliated epithelial cells of the bronchus, but not of lung tissue itself(Epstein, A.L., Marder, R.J., Winter, J.N. and Fox, R.I., 1984). It was unfortunate that all tumour samples of lung tissue were in too poor a state for immunohistochemical staining, as it is known that lung carcinoma cells with high levels of surface sialylation are much more aggressive in terms of invasion than cells with less sialylation(Ledinko, N. and Fazely, F., 1989). It has also been shown that inhibition of N-linked glycosylation of a melanoma cell-line reduces the capacity of these cells to form metastases in the lungs of injected mice(Humphries, M.J., Matsumoto, K., White, S.L. and Olden, K., 1986). This is thought to be due to a decrease in the ability of these cells to adhere to lung tissue once the cell-surface carbohydrate has been altered. Blood group antigen A on non-small cell lung cancer cells has also been shown to be a protective factor as patients of blood group A or AB who express antigen A on these cells have a much better survival rate than those who have lost Blood group A from their cell-surfaces(Lee, E.U., Roth, J. and Paulson, J.C., 1989). Blood group A is non-sialylated, but can be sialylated, and it may well be the case that sialylation of this epitope causes the "disappearance" of the epitope and at the same time increases cell-adhesiveness and therefore metastatic potential.

In summary, this small study has shown that there are differences in CDw75 expression in different tissues, and also that expression is altered in some tissues when tumours develop. There are many reports which suggest that carbohydrate changes play a major role in tumour development and metastatic potential, and so it is not surprising that CDw75 expression is altered in so many tissues. In order to determine whether the differences observed correlate with altered expression of β-galactoside-α-2,6-sialyltransferase expression, an attempt was made to produce mAbs specific for this enzyme, and these were used to immunohistochemically stain the same tissues tested here. This work will be discussed in chapter 6.

CHAPTER 5

Biochemical Analysis of CDw75.

5.1 Initial Attempts at Molecular Weight Determination.

Despite numerous attempts, the molecular weight of CDw75 has not been conclusively established. OKB4 has been reported to precipitate a band of m_r 53kDa from JOK1 cells(Dorken, B., Moller, P., Pezzutto, A., Schwartz-Albiez, R. and Moldenhauer, G., 1989), and of m_r 87kDa from Raji cells(Mittler, R.S., Talle, M.A., Carpenter, K., Rao, P.E. and Goldstein, G., 1983). EBU-141 and EBU-65 have been reported to immunoprecipitate bands of m_rs 34-38kDa and 105kDa respectively from U266 lysates(Gramatzki, M., Lauer, U., Burger, R., Huber, C., Rohwer, P., Kalden, J.R. and Henschke, F., 1989), although the bands were said to be very weak and therefore should only be considered as preliminary results. The HH2 and LN-1 epitopes have never been detected by Western blotting or immunoprecipitation. Attempts have even been made to detect the LN-1 epitope in lipid extracts from labelled Raji cells(Epstein, A.L., Marder, R.J., Winter, J.N. and Fox, R.I., 1984), but only weakly positive results were obtained.

At this point in the project, it was generally accepted that CDw75 was a cell-surface form of the enzyme β-galactoside-α-2,6-sialyltransferase(Stamenkovic, I., Asheim, H.C., Deggerdal, A., Blomhoff, H.K., Smeland, E.B. and Funderud, S., 1990) which has a predicted m_r of around 47kDa. As discussed in chapter 1, there is considerable evidence to support the existence of cell-surface glycosyltransferases and their possible roles in cell-cell interactions (reviewed in(Shur, B.D. and Roth, S., 1975; Shur, B.D., 1982)), which gives further credence to this suggestion. This being the case, CDw75 would be a glycoprotein, and it should therefore have been possible to obtain its molecular weight using standard biochemical techniques.

It is well recognised that many detergents exist, each with slightly different properties, and that the detergent used can markedly effect the integrity of the protein solubilized(Womack, M.D., Kendall, D.A. and MacDonald, R.C., 1983). Some detergents can completely denature protein whereas others are gentler and are capable of solubilisation without disrupting the structure or activity of the protein concerned. In particular it has been shown that membrane-bound enzymes are most successfully solubilized in some of the newer synthetic detergents such as CHAPS, CHAPSO, Zwittergents 310 and 312, and octylglucoside(Womack, M.D., Kendall, D.A. and MacDonald, R.C., 1983). It is possible that the structure of CDw75 is affected by different detergents and this may explain why it has never been isolated. In this chapter I therefore attempted to isolate CDw75 using a variety of detergents and

techniques in order to obtain its molecular weight and thus either add to the existing evidence for CDw75 being a cell-surface α-2,6-sialyltransferase, or disprove this theory.

5.1.1 Western Blotting Using CDw75 mAbs.

$CDw75^+$ lymphocytes from CLL patients were solubilised in 0.5% Triton CF70 and in 0.5% NP40. Lysates were diluted and boiled in reducing sample buffer and separated on 12% SDS polyacrylamide gels. Protein were electrophoretically transferred to nitro-cellulose, cut into strips and incubated with either EBU-65 or an anti-MHC class II mAb DA6-147. Bound mAb was detected using HRP-labelled goat-anti-mouse-Ig (Sigma) and colour developed using DAB as substrate.

This experiment was repeated twice, but on both occasions no bands were observed on either of the two EBU-65 stained blots, and DA6-147 was found only to have stained MHC Class II from the NP40 lysate. From this experiment, it was concluded that the structure of the EBU-65 epitope had been disrupted either by the presence of detergent, or by the reducing sample buffer and SDS PAGE.

5.1.2

Since the structure of CDw75 is altered by either SDS, reducing sample buffer or NP40, I decided to solubilise some more cells using different detergents, and to attempt to detect CDw75 by dot-blotting the lysates directly onto nitro-cellulose and staining with CDw75 mAbs. The nitro-cellulose strips were incubated in HH2, EBU-65, an IgM type CD19 mAb as a positive control and VIM13 (an IgM type CD14 mAb) as a negative control. Blots were labelled with HRP and developed as before. The results of this experiment are given in table 5.1.1 below.

Table 5.1.1 CDw75 detection by dot-blotting.

DETERGENT	HH2	EBU-65	CD19	CD14
NP40	+*	++	++	±
Triton X 100	+	++	++	±
Triton CF-54	±	+	+	±
Zwittergent	±	+	+	±

* + = moderate staining, ++ = strong staining, ± = low, background colour only.

Antigen was detected in both NP40 and Triton X 100 lysates, suggesting that the EBU-65 epitope might have been denatured during electrophoresis. I decided to try using these lysates again in SDS PAGE, using both a non-reducing sample buffer and the reducing sample buffer previously tried to determine if this was the damaging factor. However, no visible bands were detected on the Western blots from these non-reduced samples either.

5.2 Immunoprecipitation of Labelled Proteins Using CDw75 mAb.

It seemed logical at this point to attempt to label all cell-surface proteins prior to solublisation, and then to immunoprecipitate labelled CDw75 from cell lysates and identify the immunoprecipitated antigens by SDS PAGE. The advantage in using this method is that antigen recognition occurs before disruption of epitopes by SDS PAGE. Detection of previously labelled, immunoprecipitated proteins is then possible regardless of the state of the antigen after electrophoresis.

5.2.1

Lymphocytes from CLL patient 19 were biotinylated and solubilised in six different detergents, one cationic (cetylpyridinium chloride), one anionic (taurocholic acid), two non-ionic (n-octylglucoside & MEGA-8), and two zwitterionic (CHAPS & CHAPSO). Immunoprecipitations were carried out using the Protein A-Sepharose bead method, and precipitates were incubated in reducing sample buffer and run on 12% SDS polyacrylamide gels. Proteins were electrophoretically transferred to nitro-cellulose, and detected using a streptavidin-HRP conjugate and developed using E.C.L.. However, many non-specific bands were detected on the film, making it impossible to identify any specific bands. CHAPS and CHAPSO lysates were observed to contain the highest number of protein bands indicating that they were the most efficient at solubilising membrane proteins.

5.2.2

PNT cells were found to express high levels of CDw75 (Chapter 3.1.3), which made them another ideal source of antigen for biochemical studies. PNT cells were biotinylated and solubilised in CHAPS, CHAPSO and MEGA-8. Lysates were precleared by incubation with rabbit-anti-mouse-Ig (RAMG) armed beads for 1 hr,

and the supernatant decanted and used in immunoprecipitations with HH2, EBU-141, DA6-147 and DA6-231. Precipitates were heated in reducing sample buffer and run on 12% polyacrylamide gels as before, then blotted, labelled and developed using E.C.L. However, there were still too many non-specific bands detected, and no major specific bands could be seen. It was decided that further pre-clearance was necessary, and that lysates would also have to be analysed for the presence of non-denatured CDw75.

5.2.3

PNT cells were harvested from culture. Half were biotinylated and both biotinylated and nonbiotinylated cells were solubilised in 0.5% and 3% CHAPS. These lysates were used in an attempt to inhibit binding of CDw75 mAb to formaldehyde-fixed lymphocytes from CLL patient number 15. 50 µl of mAb (HH2, EBU-141 and EBU-65) were mixed with 50 µl of lysate of concentrations varying from 1/100 - neat lysate, and incubated at room temperature for 45 mins prior to addition of the fixed cells. Cells were then treated as normal for immunofluorescence labelling and analysed by flow cytometry.

The results of this experiment were difficult to analyse as the detergent itself affected fluorescence levels. This was thought to be due mainly to the disruption of the cell surface, as the forward and side scatter profiles of the detergent-exposed cells were noticeably changed. However, it did appear that HH2 binding was inhibited more by increasing concentrations of both biotinylated and unbiotinylated 0.5% CHAPS lysates.

5.2.4

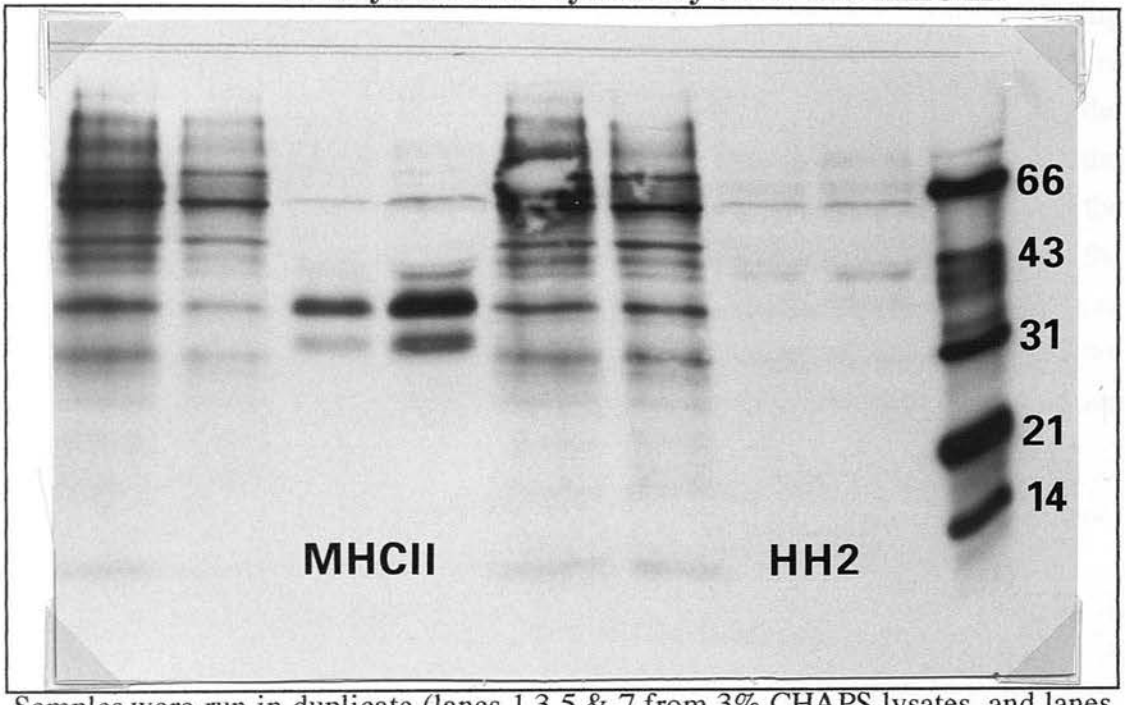
Experiment 5.2.3 was repeated using 0.5% taurodeoxycholic acid, decanesulfonic acid and digitonin lysates of biotinylated lymphocytes from CLL patient 19 to inhibit mAb binding to fixed CDw75 positive cells. However, as before, the results were indecipherable as the cells were damaged by the effects of the detergents.

5.2.5

HH2 and DA6-147 were used in an attempt to immunoprecipitate antigens from biotinylated 0.5% CHAPS PNT cell lysate. Lysates were precleared three times before immunoprecipitation. Pellets were denatured in reducing sample buffer and run on

12% polyacrylamide gels as before. Gels were Western blotted and blots labelled and developed using HRP and E.C.L. as before. The results of this experiment are shown in figure 5.2.1. As can be seen from the films, DA6-147 cleanly immunoprecipitated two proteins with m_r 33.9 kD and 29.1 kD - the expected weights for MHC Class II α and β chains. There were also two fainter bands at m_r 45.7 kD and 63.8 kD which were also found in the HH2 immunoprecipitate lanes and were suspected to be caused by the cross-reaction of human Ig with RAMG or with protein A. HH2 did not precipitate any specific bands.

Figure 5.2.1 Western blot of Antigens Immunoprecipitated from Biotinylated PNT lysates by HH2 and MHC-II.



Samples were run in duplicate (lanes 1,3,5,& 7 from 3% CHAPS lysates, and lanes 2,4,6 & 8 from 0.5% CHAPS lysates). Lanes 1 & 2 and 5 & 6 contain remaining lysate preparations after immunoprecipitation with DA6-147 and HH2 respectively.

The results of this experiment demonstrate that the immunoprecipitation system works at least for DA6-147. This is an IgG mAb, whereas the CDw75 mAbs are all of IgM isotype. It is possible that this system of immunoprecipitation does not work with IgM mAbs. It is also possible that the detergent itself interferes with the interaction between mAb and antigen, and that another detergent may be more suitable.

5.2.6

In order to check the effect of biotin on CDw75 epitope recognition, lymphocytes from CLL patient number 19 were biotinylated and immunofluorescently labelled using HH2, EBU-141, EBU-65, OKB4, DA6-147, CD14, IgG1 AFP & IgG2a AFP.

Unbiotinylated cells from this patient were stained in the same way, and fluorescence levels of the two sets of cells were compared. It was found that fluorescence levels of all biotinylated cells stained with all mAbs were no different to those of the nonbiotinylated cells. i.e. biotin does not interfere with antibody binding.

5.2.7

The mAbs HH2, OKB4 and DA6-147 were used to immunoprecipitate antigens from Digitonin lysates of biotinylated lymphocytes from CLL patient 19. Lysates were pre-cleared three times before precipitation, and precipitates were denatured in reducing sample buffer and run on 12% polyacrylamide "ready-gels" (Biorad). These were Western blotted and blots were labelled and developed using E.C.L. One of the resulting films is shown in figure 5.2.2. The film is somewhat obscured due to the stickiness of the pre-made gels. It was impossible to observe the division between the running gel and the stacking gel, and much of the stacking gel became attached to the nitro-cellulose. It can also be seen that the pre-clearance does not seem to have been as effective as previously observed, as there are still many non-specific bands. However, it can be seen that DA6-147 has precipitated the same two specific bands at 34 kD and 29 kD as in experiment 5.2.5 (figure 5.2.1).

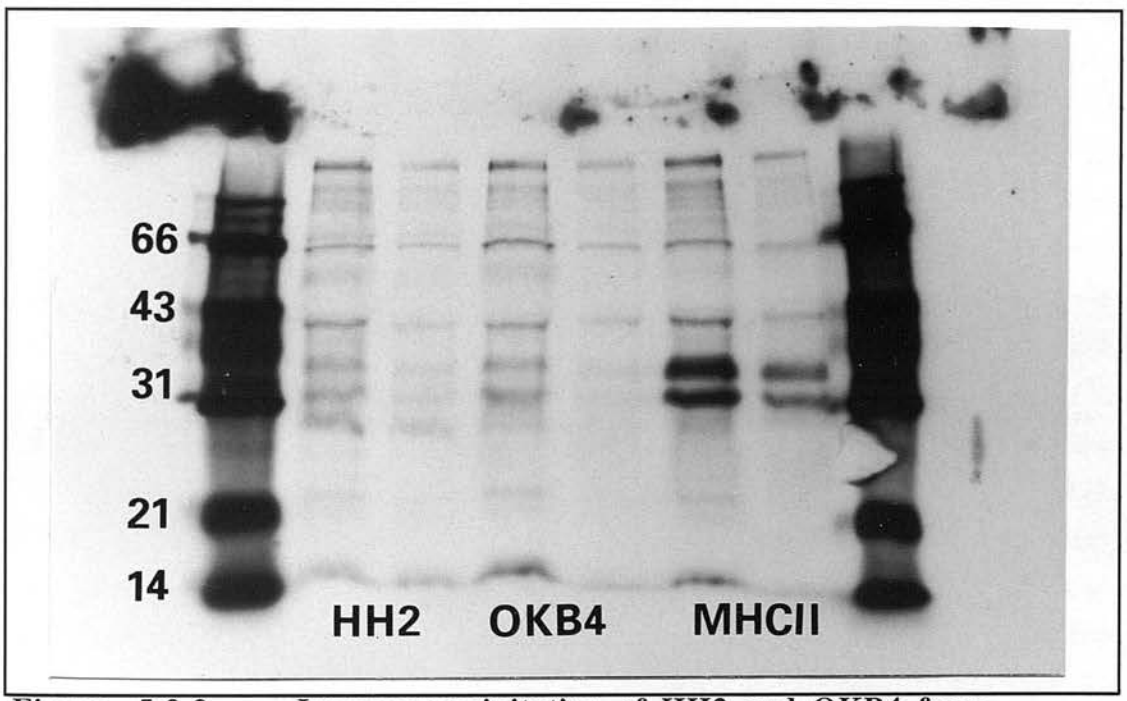


Figure 5.2.2 Immunoprecipitation of HH2 and OKB4 from biotinylated CLL cells.

The bands in the HH2 and OKB4 precipitate lanes appear to be mainly non-specific, but there is one band at m_r ~30 kD which may be specific to these immunoprecipitates as it is not seen in the DA6-147 immunoprecipitates, nor in any of the pre-clearance pellets. This experiment was repeated six times, but these were the clearest results obtained. A definitive answer is difficult without further pre-clearance to eliminate background non-specific binding.

5.3 Effects of Different Detergents on Antibody-Antigen Interactions.

A detergent may be defined as a soluble amphiphile (a molecule which is partially hydrophobic and partially hydrophilic) which effectively solubilizes membrane lipids(Keesey, J., 1987). In section 5.2 a random selection of different types of detergent were used to solubilise CDw75$^+$ cells, in the hope that one would be found which solubilized the antigen without denaturing the epitopes recognised by the CDw75 panel of mAbs. As has been described in the previous section, this approach was largely unsuccessful, and it was deemed necessary to use a larger panel of detergents and to analyse each lysate for its ability to solubilise CDw75 without denaturing the epitopes necessary for identification.

Ionic detergents although very effective at total disruption of membranes and separation of every component protein in its monomeric form, usually completely denature the protein at the same time, making them unlikely to be suitable for CDw75 purification(Keesey, J., 1987). SDS is a good example of such a detergent and its denaturing properties no doubt explain why CDw75 is not detectable after electrophoretic separation by SDS PAGE. Non-ionic detergents such as n-octyl glucoside and NP40 however, are much gentler in their solubilisation of membrane proteins. They often allow the solubilized proteins to retain native subunit structure and even enzymatic activity(Womack, M.D., Kendall, D.A. and MacDonald, R.C., 1983). Non-ionic detergents also frequently displace lipids and themselves provide a lipid-like environment to stabilise the solubilized protein. Due to their high hydrophobicity, some membrane proteins have a tendency to associate non-specifically with other proteins when solubilized by detergents. These associations can be overcome by ionic detergents, but not by non-ionic detergents. To avoid the denaturation problem of ionic detergents and at the same time prevent this non-specific protein aggregation, zwitterionic detergents such as CHAPS or CHAPSO can be used for protein solubilisation. These detergents have proved to be very effective in the

purification of other membrane-bound enzymes(Womack, M.D., Kendall, D.A. and MacDonald, R.C., 1983).

In this section a panel of twenty two different detergents were tested for their ability to inhibit binding of HH2 and EBU-141 to formaldehyde-fixed lymphocytes from CLL patient 15. mAb binding was detected using a radio-immunoassay as an alternative to flow cytometry, as this is not affected by the damage to the fixed cell surface caused by detergent. Once it had been established which detergents did not interfere with mAb binding, these were used to make CDw75$^+$ cell lysates which were in turn also tested for their ability to inhibit CDw75 mAb binding.

Table 5.3.1 Panel of detergents used for inhibition studies.

Detergent name	No.	Type	Source
Cetyl pyridinium chloride	1	cationic	Sigma
Taurocholic acid	2	anionic	Sigma
N-octyl glucoside	3	non-ionic	Sigma
NP40	4	non-ionic	Sigma
CHAPS	5	zwitterionic	Boehringer Mannheim
CHAPSO	6	zwitterionic	Boehringer Mannheim
Taurodeoxycholic acid	7	anionic	Sigma
Decanesulfonic acid	8	anionic	Sigma
Triton WR-1339	9	non-ionic	Sigma
Triton CF-54	10	non-ionic	Sigma
Triton X-100	11	non-ionic	Sigma
Triton X-114	12	non-ionic	Sigma
N-dodecyl-N,N-dimethyl-3-ammonio-1-propane sulphonate	13	zwitterionic	Sigma
N-dodecyl-β-D-maltoside	14	non-ionic	Sigma
Isotridecylpoly(ethylene glycol)$_n$	15	non-ionic	Boehringer Mannheim
MEGA-8	16	non-ionic	Boehringer Mannheim
Thesit	17	non-ionic	Boehringer Mannheim
Dodecyltrimethylammonium bromide	18	cationic	Sigma
N-dodecylglucoside	19	non-ionic	Sigma
Digitonin	20	non-ionic	Sigma
MEGA-10	21	non-ionic	Boehringer Mannheim
Hexadecyltrimethylammonium bromide	22	cationic	Sigma

5.3.1 CDw75 Radioimmunoassay of Detergent-treated Cells.

Fixed lymphocytes from CLL patient 15 were incubated in HH2 or EBU-141 (at the minimum concentration necessary for detectable labelling) in the presence of 0.5% detergent. A panel of 22 different detergents of various types (as described in table 5.3.1) were used. mAb binding was detected using a radiolabelled secondary mAb in a radioimmunoassay. This experiment was repeated three times.

Table 5.3.2 Results of Radioimmunoassay of Detergent-treated Cells.

Detergent No.	HH2 (cpm)	HH2 (% of normal*)	EBU-141 (cpm)	EBU-141 (% of normal)	Inhibition
1	1084	9	1646	15	+++
2	822	3	4560	60	++
3	3848	70	6666	93	+
4	212	0	278	0	++++
5	5848	≥ 100	6578	92	none
6	6026	≥ 100	6960	98	none
7	2060	31	1498	13	+++
8	2620	43	5736	79	++
9	4492	84	5962	82	±
10	654	0	880	3	++++
11	560	0	646	0	++++
12	340	0	270	0	++++
13	366	0	534	0	++++
14	254	0	320	0	++++
15	468	0	496	0	++++
16	3984	73	5026	68	+
17	362	0	708	1	++++
18	690	0	3382	42	+++
19	1730	23	1572	14	+++
20	6124	≥ 100	7892	≥ 100	none
21	2810	47	4592	61	++
22	856	4	1270	9	+++
NONE	5197	100	7109	100	none

* % of normal cpm in the absence of detergent after adjusting for background.

The results of each experiment indicated similar levels of binding inhibition by respective detergents. Representative results from one experiment are given in table 5.3.2.

The results of this experiment indicate that the detergents which cause least interference to mAb binding are Digitonin, CHAPSO, CHAPS, N-octylglucoside, Triton WR-1339, MEGA-8, MEGA-10, Decanesulfonic acid and Taurocholic acid.

5.3.2 Determination of Epitope Structure Preservation in Non-inhibitory Detergents.

In order to determine which detergents solubilised CDw75 without disrupting the epitopes recognised by the available mAbs, PNT cells were biotinylated and solubilised in 0.5% Digitonin, CHAPSO, CHAPS, N-octylglucoside, Triton WR-1339, MEGA-8, MEGA-10 and Taurocholic acid. These lysates and samples of 0.5% detergent alone were used to inhibit binding of HH2 and EBU-141 to fixed lymphocytes from CLL patient 15. Binding was detected by radioimmunoassay in the same way as before (5.3.1). Each inhibition was carried out in triplicate, and the mean cpm after adjustment for background levels of radioactivity (as determined by binding of IgM anti-CD14 mAb VIM13) are given in table 5.3.3 below.

Table 5.3.3 Results of Lysate Inhibition of CDw75 Binding.

Detergent No.	Detergent	Lysate	% Inhibition	Detergent	Lysate	% Inhibition
	H H 2			**E B U-1 4 1**		
2	4268	1112	74	3150	1886	40
3	3774	396	90	5444	504	91
5	2124	236	89	5498	814	85
6	4948	2282	54	4538	800	82
9	4296	1502	65	5010	3214	36
16	3932	0	100	4938	2826	43
20	6376	864	86	5434	2342	57
21	2198	894	59	2592	1036	60
NONE	3908	3908	0	5547	5547	0

These results indicate that intact, non-denatured CDw75 is present in all of the lysates tested. Detergent numbers 3, 5, 16 and 20 were observed to preserve both HH2 and

EBU-141 epitopes most successfully. These are N-octylglucoside, CHAPS, MEGA-8 and Digitonin respectively, i.e. one zwitterionic and three non-ionic detergents. These detergents should make the best candidates for use in cell lysis for immunoprecipitation. The amalgamated results of section 5.3 are summarised in table 5.3.4 below.

Table 5.3.4 Summary of Results of Section 5.3.

DETERGENT	No.	mAb Binding	Epitope Preservation
Cetyl pyridinium chloride	1	inhibited	n.d.*
Taurocholic acid	2	slightly inhibited	disrupted
N-octyl glucoside	3	normal	preserved
NP40	4	inhibited	n.d.
CHAPS	5	normal	preserved
CHAPSO	6	normal	disrupted
Taurodeoxycholic acid	7	inhibited	n.d.
Decanesulphonic acid	8	slightly inhibited	n.d.
Triton WR-1339	9	slightly inhibited	disrupted
Triton CF-54	10	inhibited	n.d.
Triton X-100	11	inhibited	n.d.
Triton X-114	12	inhibited	n.d.
N-dodecyl-N,N-dimethyl-3-ammonio-1-propane sulphonate	13	inhibited	n.d.
N-dodecyl-β-D-maltoside	14	inhibited	n.d.
Isotridecylpoly(ethylene glycol)$_n$	15	inhibited	n.d.
MEGA-8	16	slightly inhibited	HH2 preserved
Thesit	17	inhibited	n.d.
Dodecyltrimethylammonium bromide	18	inhibited	n.d.
N-dodecylglucoside	19	inhibited	n.d.
Digitonin	20	normal	preserved
MEGA-10	21	slightly inhibited	disrupted
Hexadecyltrimethylammonium bromide	22	inhibited	n.d.

* n.d. = not determined.

5.4 Final Attempts at Immunoprecipitation of CDw75.

Using the information gained about the various detergents, lysates were made using detergents least likely to disrupt the CDw75 epitopes, and further attempts were made to immunoprecipitate CDw75 using a variety of techniques in an attempt to formally identify "CD75" by determining its molecular weight.

5.4.1 Protein-A Sepharose CDw75 Immunoprecipitations.

Lysates were made of biotinylated PNT cells, using N-octylglucoside, CHAPS, Digitonin and MEGA-8. These were used in immunoprecipitations with the mAbs HH2, OKB4 and DA6-147. The precipitated pellets and whole lysates were reduced in sample buffer, run on 12% gels and transferred to nitro-cellulose by Western blotting. Bands were labelled with HRP and detected using E.C.L.

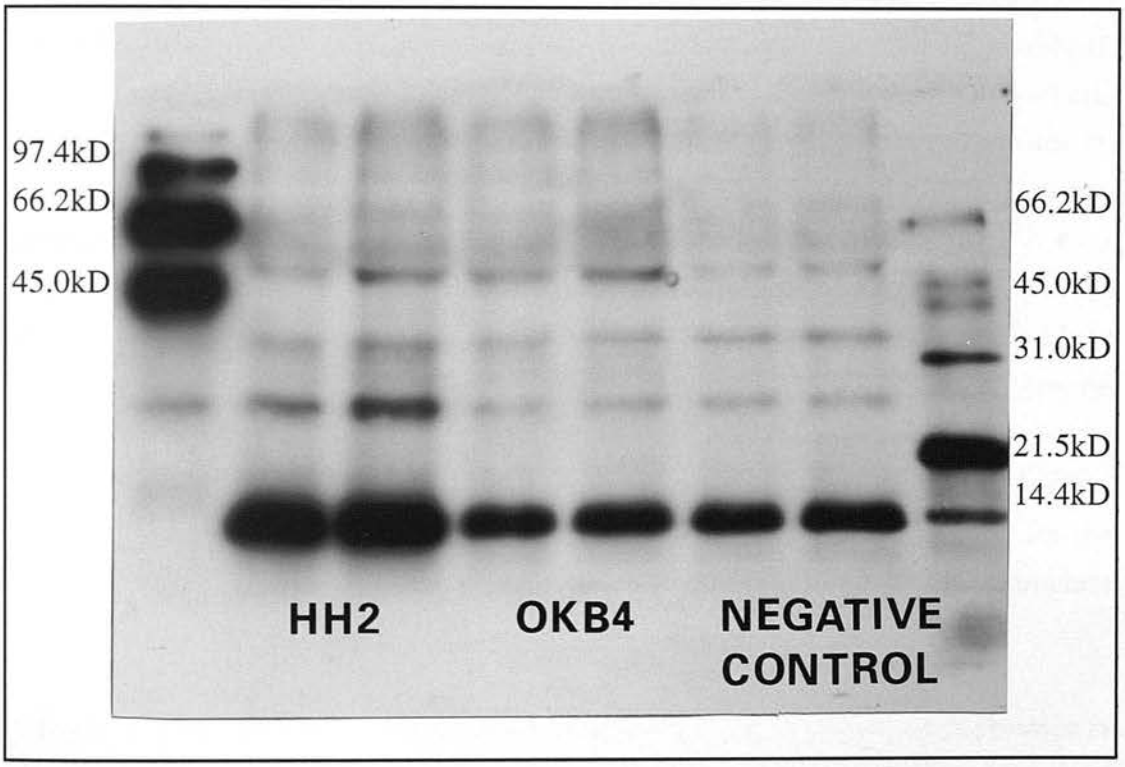
One major band of m_r ~34 kD was detected in all immunoprecipitates from N-octylglucoside lysates (not shown). This was also visible as a double band at the same molecular weight in all immunoprecipitates from MEGA-8 lysates. In CHAPS lysates the same band was detected in the HH2 immunoprecipitate, and was present as a double band in the DA6-147 precipitate. No bands were detected in the OKB4 immunoprecipitate from CHAPS. The pellets from the pre-clearance steps were also analysed by SDS PAGE and Western blotting, and this same band was weakly detected in the pre-clearance pellets from the above three detergent lysates. No specific bands were detected in any of the Digitonin lysate immunoprecipitates. This was thought to be due to the precipitation of many proteins with Digitonin itself which became insoluble when mixed with aqueous buffers.

The 34 kD band did not appear to be specific as it was present in DA6-147 immunoprecipitates as well as CDw75 precipitates, unless CDw75 is around the same molecular weight as MHC Class II. It was therefore impossible to draw any definite conclusions from this experiment.

5.4.2 CDw75 Immunoprecipitation Using Dynabeads.

Tosylactivated Dynabeads (Dynal) were covalently attached to goat-anti-mouse-IgM, and used to immunoprecipitate CDw75 (using HH2 and OKB4), CD20 (using an IgM mAb 019 B-C1 from the 3rd Leucocyte Typing Workshop) and CD21 (using an IgM mAb B46 -B2 from the 4th Leucocyte Typing Workshop) from water soluble Digitonin lysates of biotinylated lymphocytes from CLL patients 18 and 24. Immunoprecipitates were reduced in sample buffer and run on 12% SDS Polyacrylamide gels, transferred to nitro-cellulose by Western blotting, HRP labelled, and developed using E.C.L. The developed films are shown in figure 5.4.1.

Figure 5.4.1 Dynabead immunoprecipitated CDw75 from biotinylated Digitonin CLL lysates.



As can be seen from figure 5.4.1, a single dominant band was detected in CDw75 immunoprecipitates at a molecular weight of ~ 14 kD. However, this band was also detected in the negative control , and appeared to be the major constituent of the whole lysate. Other weaker bands were also detected, but these too were found to be non-specific. No specific bands were detected at m_r 35 and 140 kD in the CD20 and CD21 positive controls (not shown) either, contrary to expectations. It was therefore concluded that the affinity purified anti-IgM had not bound to the primary mAbs, and

that all bands detected were due to non-specific binding of anti-IgM-coated beads to major proteins in the lysates.

5.5 Discussion.

As discussed in 5.1, all previous attempts at isolating and definitively identifying CDw75 by biochemical means have been unsuccessful. Despite my rigorous efforts using different detergents and even different means of immunoprecipitation, CDw75 was not isolated. CDw75 was undetectable after SDS PAGE and Western blotting, and I was unable to immunoprecipitate any specific antigens from any detergent lysates using these IgM mAbs. Unless an IgG mAb could be produced, I was unlikely to obtain any biochemical information about this elusive antigen. At this point in the project, it was still universally accepted that CDw75 was most likely to be a cell-surface sialyltransferase(Stamenkovic, I., Asheim, H.C., Deggerdal, A., Blomhoff, H.K., Smeland, E.B. and Funderud, S., 1990). I therefore decided to have synthetic peptides constructed from parts of the known amino acid sequence of ß-galactoside-α-2,6-sialyltransferase, and to use these as immunogens in order to raise IgG mAbs against CDw75, and this work is discussed in chapter 6.

However, since this work was completed, it has been proved that CDw75 is unlikely to be a cell-surface sialyltransferase and is more likely to be a carbohydrate moiety on the cell-surface that is generated by the intracellular activity of the enzyme β-galactoside-α-2,6-sialyltransferase(Munro, S., Bast, B.J., Colley, K.J. and Tedder, T.F., 1992). Since this information came to light, a more logical explanation for the lack of success described above may be that CDw75 is in fact a carbohydrate structure or ganglioside attached to the lipid bilayer of the cell-surface.

Gangliosides are expressed ubiquitously on all cells and their pattern of expression is developmentally regulated. They are mainly located on the outer leaflet of the lipid bilayer, and composed of hydrophilic carbohydrate chains which are linked to the lipid by a covalent link with ceramide(Yuasa, H., Scheinberg, D.A. and Houghton, A.N., 1990). Ceramide is the hydrophobic part of the complex which inserts into the plasma membrane. It is made up of a sphingoid base attached to a long chain fatty acid. The initial step in synthesis of the glycosphingolipids involves the attachment of a glucose or galactose residue to ceramide by a glycosidic β-linkage to the primary hydroxyl group of the sphingosine. For the synthesis of gangliosides, a galactose residue is

then linked to glucose to form lactosyl-ceramide. This represents the core structure of most glycosphingolipids and is designated Galβ-1,4-Glcβ-1,1-Cer. The addition of further sugar groups to this core structure leads to the formation of five different series of glycosphingolipids. Some gangliosides bear terminal Galβ-1,4-GlcNAc groups which can be sialylated by either α-2,3- or α-2,6-sialyltransferase. The α-2,6-linked sialylated gangliosides are less common than the α-2,3-linked residues, but they have been identified on human lymphoid tissue(Kiguchi, K., Henning-Chubb, C. and Huberman, E., 1993). Expression of these gangliosides is dependant on the regulation of the genes encoding the glycosyltransferases involved, and it is widely accepted that the same glycosyltransferase enzymes are capable of glycosylating both glycoproteins and glycolipids(Hakomori, S., Patterson, C.M., Nudelman, E. and Sekiguchi, K., 1983; Feizi, T., 1985; Dall'Olio, F., Malagolini, N., di Stefano, G., Minni, F., Marrano, D. and Serafini-Cessi, F., 1989; Paulson, J.C. and Colley, K.J., 1989; Kanani, A., Sutherland, D.R., Fibach, E., Matta, K.L., Hindenburg, A., Brockhausen, I., Kuhns, W., Taub, R.N., van den Eijnden, D.H. and Baker, M.A., 1990; Erikstein, B.K., Funderud, S., Beiske, K., Aas-Eng, A., De Lange Davies, C., Blomhoff, H.K. and Smeland, E.B., 1992; Shah, S., Lance, P., Smith, T.J., Berenson, C.S., Cohen, S.A., Horvath, P.J., Lau, J.T. and Baumann, H., 1992).

Further evidence for the theory that CDw75 may be a glycolipid can be found in lymphocyte activation studies. As shown in chapter 3, when normal human T-cells are stimulated with PHA, CDw75 expression is increased. It has also been shown that phorbol esters lead to increased α-2,6-sialyltransferase activity in the human pro-myelocytic leukaemia cell line HL-60(Kiguchi, K., Henning-Chubb, C. and Huberman, E., 1993), and in Chinese hamster V79 cells(Burczak, J.D., Moskal, J.R., Trosko, J.E., Fairley, J.L. and Sweeley, C.C., 1983). In addition, it has been shown that expression of sialylated gangliosides is increased when lymphocytes are stimulated with PHA(Basu, S.K., Whisler, R.L. and Yates, A.J., 1986). These changes are consistent both with the theory that CDw75 is controlled by the expression of α-2,6-sialyltransferase, and that the antigen may be present on glycolipids.

In section 5.3 it was observed that 8/8 detergents tested did not denature the epitopes recognised by HH2 and EBU-141, and yet when these detergents were used to solubilise CDw75$^+$ cells (in section 5.4), no antigen was detected after electrophoretic separation of CDw75 immunoprecipitates. At the time, this was thought to be due to the inability of the IgM CDw75 mAbs to immunoprecipitate antigen. However, if

CDw75 is not present on cell-surface glycoproteins to any great extent, and instead is present mainly on glycolipid, no antigen would have been detected after electrophoresis as glycolipid would not be labelled by the biotinylation technique employed, nor would it be separated by electrophoresis.

Other workers have also suggested that CDw75 may be expressed predominantly on glycolipid as the CDw75 antigen is resistant to formalin fixation and paraffin embedding - processes which often denature cell-surface proteins(Bast, B.J., Zhou, L.J., Freeman, G.J., Colley, K.J., Ernst, T.J., Munro, J.M. and Tedder, T.F., 1992; Munro, S., Bast, B.J., Colley, K.J. and Tedder, T.F., 1992). CD76 has also been shown to be such a glycolipid, whose expression is regulated by the same intracellular β-galactoside-α-2,6-sialyltransferase(Munro, S., Bast, B.J., Colley, K.J. and Tedder, T.F., 1992). One report also states that solid phase radioimmunoassays of Raji cell lipid extracts gave weakly positive results, although TLC grams of these extracts failed to give positive results when probed with LN-1. Perhaps further research in this area is required in order to determine if the CDw75 antigen is in fact a component of cell-surface glycolipid.

The role of carbohydrate on both glycoproteins and lipids on the cell-surface is increasingly being recognised as one of great importance. The level of control over cell-cell interactions via specifically glycosylated receptors is immense as each individual glycosidic linkage is controlled by a separate enzyme. If we consider sialic acid alone, there are seven specific sialyltransferases necessary for synthesis of the following different attachments(Joziasse, D.H., Bergh, M.L.E., Hart, H.G.J.t., Koppen, P.L., Hooghwinkel, G.J.M. and Van den Eijnden, D.H., 1985):

α-2,3-Galβ-1,3-GalNAc; α-2,6-Galβ-1,3-GalNAc;
α-2,3-Galβ-1,3/4-GlcNAc; α-2,6-Galβ-1,4-GlcNAc;
α-2,4-Galβ-1,4-GlcNAc; α-2,6-Galβ-1,3-GlcNAc;
and α-2,8-NeuAcα-2,3-Gal.

If we also consider that there are many other sugar residues such as glucose, N-acetyl glucosamine, fucose, galactose and galactosamine, all of which are in turn controlled by several transferase enzymes, we begin to appreciate the level of control and the specificity of carbohydrate structures on the cell-surface and in the circulation. The presence of carbohydrate can transform the surface of a cell and completely change its interactions with other cells and circulating cytokines. Carbohydrates are also

hydrophilic, charged moieties, and as a result, their presence on the cell-surface can therefore alter the charge of the cell which may also influence its interactions.

CDw75 has been proposed to act as a receptor on B-cells for CD22β on other B-cells(Stamenkovic, I., Sgroi, D., Aruffo, A., Sy, M.S. and Anderson, T., 1991). Interaction with CD22β$^+$ cells is therefore only possible when the enzyme β-galactoside-α-2,6-sialyltransferase is expressed and active in receptor B-cells. An additional level of control over CDw75 interaction must also be possible since different sized mRNA transcripts for a single β-galactoside-α-2,6-sialyltransferase gene have been detected in different cell types(Aasheim, H.C., Aas-Eng, D.A., Deggerdal, A., Blomhoff, H.K., Funderud, S. and Smeland, E.B., 1993). The differences appear to be in the 5' untranslated region of the same gene, indicating that alternate promoter sites operate in a complex level of additional control over sialylation of glycoproteins and glycolipids. Thus even though CDw75 is not a cell-surface β-galactoside-α-2,6-sialyltransferase, and is likely to be a carbohydrate moiety on cell-surface glycolipids rather than on glycoproteins, it may still be of major importance in many processes such as protein targeting, cell-cell interactions including growth regulation and therefore in tumour progression, and in developmental processes. Indeed, elevated levels of α-2,6-sialylated glycolipids have been observed in some cancers(Hakomori, S., Patterson, C.M., Nudelman, E. and Sekiguchi, K., 1983).

CHAPTER 6

Production of Monoclonal Antibodies Against α-2,6-Sialyltransferase.

6.1 Protein Structure Prediction for α-2,6-Sialyltransferase.

As mentioned in Chapter 1, the enzyme β-galactoside-α-2,6-sialyltransferase was first isolated and characterised from bovine colostrum in 1973(Bartholomew, B.A., Jourdian, G.W. and Roseman, S., 1973). Since then, it has been found in a variety of tissues and in many different species. In rat where it has been most extensively studied, five different mRNA transcripts for the enzyme have been described, all arising from a single gene(Wang, X., O'Hanlon, T.P., Young, R.F. and Lau, J.T., 1990; Wen, D.X., Svensson, E.C. and Paulson, J.C., 1992). However, much less is known about the enzyme in human tissues, and different isoforms have yet to be identified.

The cDNA from the human form of the enzyme has been sequenced(Lance, P., Lau, K.M. and Lau, J.T., 1989; Grundmann, U., Nerlich, C., Rein, T. and Zettlmeissl, G., 1990; Stamenkovic, I., Asheim, H.C., Deggerdal, A., Blomhoff, H.K., Smeland, E.B. and Funderud, S., 1990), and has been shown to share 80-87% homology with the amino acid sequence of rat β-galactoside-α-2,6-sialyltransferase. The enzyme described by Stamenkovic *et al* is a 405 residue polypeptide (as illustrated in Figure 6.1.1) with a predicted m_r of 46.367 kDa. Like other glycosyltransferase enzymes, the human form of this sialyltransferase consists of a short 9 amino acid NH_2-terminal domain which is thought to be cytoplasmic followed by a series of 17-18 hydrophobic residues which are thought to constitute a transmembrane domain of the enzyme. The extracellular domain (or lumenal domain in Golgi expression) of 378 residues is catalytically active(Stamenkovic, I., Asheim, H.C., Deggerdal, A., Blomhoff, H.K., Smeland, E.B. and Funderud, S., 1990). In rats, this domain has been detected on its own as a soluble form of the enzyme in plasma and colostrum(Nemansky, M. and Van den Eijnden, D.H., 1992). It is likely that proteolytic cleavage of the catalytic site also occurs in humans as enzyme activity has been detected in human plasma and colostrum(Bartholomew, B.A., Jourdian, G.W. and Roseman, S., 1973).

As with the work described in the previous chapter, production of mAbs against human β-galactoside-α-2,6-sialyltransferase was initiated at a stage in the project when it was believed that CDw75 was a cell-surface form of this enzyme. The main aim of this work was to produce mAbs against the protein component of this glycoprotein which were of IgG isotype in order to compare tissue distribution with the original CDw75 mAbs and thus to provide further evidence for the proposed

identity of the antigen. The new IgG mAbs would also be used in biochemical characterisation of CDw75 in the hope that these mAbs would successfully immunolabel the antigen on electrophoretically separated preparations of solubilised membrane proteins, or be able to immunoprecipitate it from such samples. However, before antibody production was completed, as previously mentioned, it became evident that CDw75 is more likely to be a carbohydrate moiety on the cell-surface which is generated by the activity of this enzyme(Munro, S., Bast, B.J., Colley, K.J. and Tedder, T.F., 1992). Since production of the mAbs was already well underway, it was decided to continue with the experiment and to use the β-galactoside-α-2,6-sialyltransferase-specific mAbs to compare the tissue distributions of CDw75 and the α-2,6-sialyltransferase enzyme.

There are various methods of predicting antigenic sites on proteins(Stern, P.S., 1991). Epitopes were originally classed as either sequential or conformational meaning that antigenicity was dependent only upon the amino acid sequence or that it was dependent on the actual shape of the epitope. Subsequently, hydrophilicity was used as a measure of antigenicity, as the most hydrophilic residues are more likely to be on external domains of the protein and are thus more exposed to the immune system. Mobility of particular domains was another indication of immunogenicity, but by definition, the most mobile domains of a protein are most likely to be external domains and thus be hydrophilic. Other methods used have involved protrusion indices, accessibility and hydropathicity measurements. In general however, the most reliable methods of predicting antigenicity involve a combination of the above.

6.1.1 Synthetic Peptide Production.

In this study, a computer structure prediction program written by E.E.Eliopoulos, (Secondary Structure Prediction Suite Version 2.2, March 1986) was used to predict the three-dimensional structure of the enzyme. The program searches for particular amino acid motifs in order to predict domains of the protein which are most likely to be helical, β-sheets, turns or hydrophobic regions. The results of this prediction method are illustrated in figure 6.1.2. From the amino acid sequence of the enzyme (listed in figure 6.1.1)(Stamenkovic, I., Asheim, H.C., Deggerdal, A., Blomhoff, H.K., Smeland, E.B. and Funderud, S., 1990), it was also possible to predict all potential N- and O-linked glycosylation sites.

Figure 6.1.1 Amino Acid Sequence of Human β-Galactoside α-2,6-Sialyltransferase.

1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20 21 22 23 24 25 26 27 28 29 30
M I H T N L K K K F S C C V L V F L L F A V I C V W L E K K

31 32 33 34 35 36 37 38 39 40 41 42 43 44 45 46 47 48 49 50 51 52 53 54 55 56 57 58 59 60
K G S Y Y D S F K L Q T K E F Q V L K S L G K L A M G S D S

61 62 63 64 65 66 67 68 69 70 71 72 73 74 75 76 77 78 79 80 81 82 83 84 85 86 87 88 89 90
Q S V S S S S T Q D P T G R Q T L G S L R G L A K A K P E A

91 92 93 94 95 96 97 98 99 100 101 102 103 104 105 106 107 108 109 110 111 112 113 114 115 116 117 118 119 120
S F Q V W N K D S S S K N L I P R L Q K I W K N Y L S M N K

121 122 123 124 125 126 127 128 129 130 131 132 133 134 135 136 137 138 139 140 141 142 143 144 145 146 147 148 149 150
Y K V S Y K G P G P G I K F S A E A L R C H L R D H V N V S

151 152 153 154 155 156 157 158 159 160 161 162 163 164 165 166 167 168 169 170 171 172 173 174 175 176 177 178 179 180
M V E V T D F P F N T S E W E G Y L P K E S I R T K A G P W

181 182 183 184 185 186 187 188 189 190 191 192 193 194 195 196 197 198 199 200 201 202 203 204 205 206 207 208 209 210
G R C A V V S S A G S L K S S Q L G R E I D D H D A V L R F

211 212 213 214 215 216 217 218 219 220 221 222 223 224 225 226 227 228 229 230 231 232 233 234 235 236 237 238 239 240
N G A P T A N F Q Q D V G T K T T I R L M N S Q L V T T E K

241 242 243 244 245 246 247 248 249 250 251 252 253 254 255 256 257 258 259 260 261 262 263 264 265 266 267 268 269 270
R F L K D S L Y N E G I L I V W D P S V Y H S D I P K W Y Q

271 272 273 274 275 276 277 278 279 280 281 282 283 284 285 286 287 288 289 290 291 292 293 294 295 296 297 298 299 300
N P D Y N F F N N Y K T Y R K L H P N Q P F Y I L K P Q M P

301 302 303 304 305 306 307 308 309 310 311 312 313 314 415 316 317 318 319 320 321 322 323 324 325 326 327 328 329 330
W E L W D I L Q E I S P E E I Q P N P P S S G M L G I I I M

331 332 333 334 335 336 337 338 339 340 341 342 343 344 345 346 347 348 349 350 351 352 353 354 355 356 357 358 359 360
M T L C D Q V D I Y E F L P S K R K T D V C Y Y Y Q K F F D

361 362 263 364 365 366 367 368 369 370 371 372 373 374 375 376 377 378 379 380 381 382 383 384 385 386 387 388 389 390
S A C T M G A Y H P L L Y E K N L V K H L N Q G T D E D I Y

391 392 393 394 395 396 397 398 399 400 401 402 403 404 405
L L G K A T L P G F R T I H C

N X S/T represent possible N-linked glycosylation sites, possible O-linked sites are indicated by S and T, and peptide amino acid sequences are indicated by SHADING

Figure 6.1.2 illustrates the results of the protein structure prediction. As can be seen from this chart, there appear to be three major turns (highlighted) on the protein which are also hydrophilic and which do not contain any possible glycosylation sites. The sequences of these three regions were chosen for the synthesis of peptide immunogens, as hydrophilic turns are most likely to be on external domains of the protein. It was also desirable to use regions of the protein which would not normally be masked by carbohydrate, as this may prevent binding of any mAbs raised to the enzyme in its native state. The amino acid sequences of the three peptides chosen are listed in table 6.1.1.

FIGURE 6.1.2 PROTEIN STRUCTURE PREDICTION FOR HUMAN β-GALACTOSIDE α-2,6-SIALYLTRANSFERASE

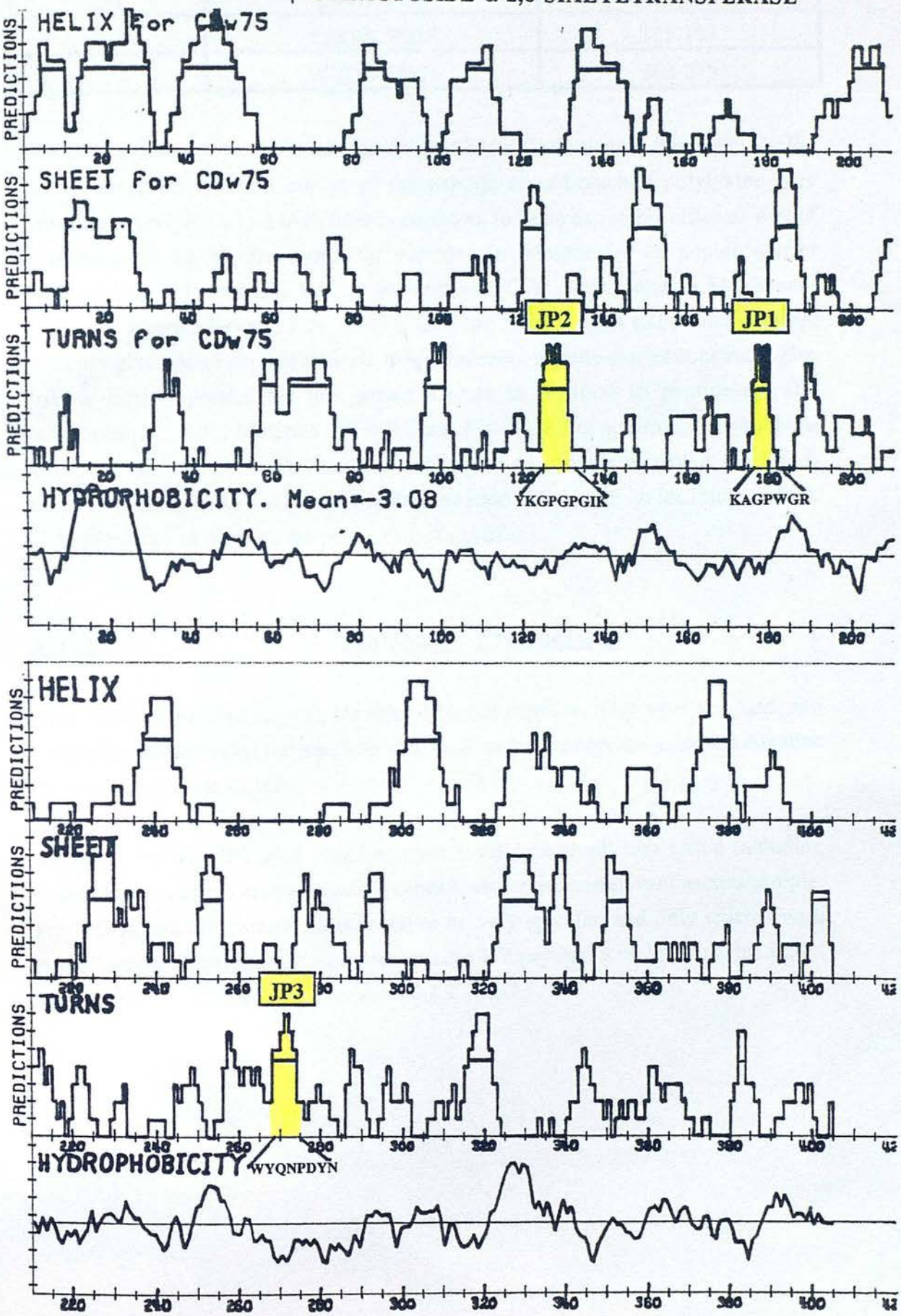


Table 6.1.1 Amino acid sequences of synthetic peptides.

PEPTIDE	SEQUENCE	RESIDUE NUMBERS
JP1	KAGPWGR	176-182
JP2	YKGPGPGIK	125-133
JP3	WYQNPDYN	268-275

The peptides were constructed in the form of Multiple Antigenic Peptides (MAPs) which consisted of eight copies of the peptide on a branched polylysine core (illustrated in figure 2.1). MAPs have been shown to be an extremely efficient way of producing mAbs against synthetic peptides in comparison to hapten-carrier systems(Tam, J.P. and Lu, Y.A., 1989; McLean, G.W., Owsianka, A.M., Subak-Sharpe, J.H. and Marsden, H.S., 1991). They are large enough to be immunogenic and only give rise to peptide-specific mAbs, whereas peptide-carrier constructs give rise to mAbs specific for the protein carrier in addition to peptide-specific mAbs(Posnett, D.N., McGrath, H. and Tam, J.P., 1988). In addition, the problems of peptide damage by harsh chemical conjugation procedures to protein carriers is avoided by using MAPs. All three MAPs were used individually in the immunisation of three batches of mice for the production of mAbs.

6.1.2 Antibody Production.

After three immunisations with the three different peptides, mice were test bled, and the serum samples tested for reactivity with their respective peptides using the Alkaline Phosphatase peptide ELISA.

Peptides JP1 and JP2 were found to react strongly with all sera tested including serum from a normal mouse negative control, and with an irrelevant ascites sample. The JP3 peptide in contrast, was found to be very specific, and only reacted with serum samples from mice immunised with the JP3 peptide. Results from this initial screening of 12 mice are given in tables 6.1.2 - 6.1.4.

Table 6.1.2 **JP1 ELISA Results.**

	Absorbance at 405 nm					
	Serum Concentration					
MOUSE	1/100	1/200	1/400	1/800	1/1600	1/3200
control	0.633	1.471	>2	>2	>2	>2
1	1.883	>2	>2	>2	>2	>2
2	1.240	>2	>2	>2	>2	>2
3	1.469	>2	>2	>2	>2	>2
4	>2	>2	>2	>2	>2	>2
OKB4 asc*	1.488	>2	>2	>2	>2	>2

* asc = ascites

Table 6.1.3 **JP2 ELISA Results.**

	Absorbance at 405 nm					
	Serum Concentration					
MOUSE	1/100	1/200	1/400	1/800	1/1600	1/3200
control	0.231	0.921	>2	>2	>2	>2
1	1.056	1.621	>2	>2	>2	>2
2	0.594	1.567	>2	>2	>2	>2
3	0.577	>2	>2	>2	>2	>2
OKB4 asc	0.767	1.309	>2	>2	>2	>2

Table 6.1.4 **JP3 ELISA Results.**

	Absorbance at 405 nm					
	Serum Concentration					
MOUSE	1/100	1/200	1/400	1/800	1/1600	1/3200
control	0.269	0.265	0.244	0.192	0.083	0.111
1	0.980	0.793	0.539	0.441	0.194	0.187
2	0.651	0.384	0.292	0.233	0.140	0.101
3	0.593	0.472	0.446	0.300	0.190	0.126
4	0.812	0.572	0.472	0.346	0.291	0.177
5	0.607	0.367	0.334	0.257	0.152	0.133
OKB4 asc	0.049	0.025	0.038	0.050	0.006	0.067

From these results it would appear that peptides JP1 and JP2 react non-specifically with the alkaline phosphatase-labelled anti-mouse-Ig antibody, and are thus of no use in this assay. It was therefore decided to use only JP3 immunised mice in the synthesis of monoclonal antibodies against sialyltransferase.

6.1.3 Hybridoma Screening.

After booster injections of JP3, mice were again test-bled and the spleen of the mouse with the highest antibody titre was used in the production of mAbs against sialyltransferase. Hybridomas were screened by testing cell culture supernatants using the JP3 ELISA and the RAJI cell-bound ELISA. Cells with supernatants which specifically bound to JP3 and exhibited peri-nuclear staining of RAJI cells were selected, cloned and cultured in larger volumes in order to produce more cells. Samples of cells from all cultures were also frozen down in liquid nitrogen for future use. Supernatants from these cultures were re-tested by JP3 ELISA and Raji-cell-bound ELISA. The results of these ELISAs are given in table 6.1.5 below.

In the JP3 ELISA, the mean of the negative control values + 3 x standard deviation was 0.069 absorbance units. Cells in wells 3H12, 3C11, 1A6, 3F11, 2D5, 4G1, 3C8 and 2D8 were found to secrete significant levels of JP3-specific Ab. These cells were all re-cloned at this stage. Supernatants from 1F9 and 3E10 were weakly positive and were also cloned in the hope that a strong mAb secreting clone would emerge. Another cell culture in 3G1 was observed to be growing well at this stage and this was also cloned.

Table 6.1.5 Results of JP3 and RAJI cell-bound ELISA's of JP3 hybridoma supernatants.

CULTURE WELL REF.	RAJI ELISA	A_{490} (JP3 ELISA)	CULTURE WELL REF.	RAJI ELISA	A_{490} (JP3 ELISA)
3H12	++	0.245	**2D5**	++	0.610
3C11	++	0.116	**4G1**	++	0.241
1A6	+++	0.763	**3E10**	+	0.066
4A7	-	0.026	**3C8**	++	0.124
3F11	++	0.244	**1F9**	+	0.033
4A1	±	0.030	**2D8**	++	0.266

The hybridomas produced from this fusion were named the Pst series (possible sialyltransferase). Clones were re-named as described in table 6.1.6 below, and supernatants from all 12 sets of clones were tested three days later using the RAJI cell-bound ELISA. At least one positive supernatant was obtained from each set.

Table 6.1.6 New Clone Names.

OLD NAME	4G1	1A6	1F9	2D5	2D8	3C8
NEW NAME	Pst 1.1-8	Pst 2.1-8	Pst 3.1-8	Pst 4.1-8	Pst 5.1-8	Pst 6.1-8
OLD NAME	3C11	3E10	3F11	3G1	3H12	4A1
NEW NAME	Pst 7.1-8	Pst 8.1-8	Pst 9.1-8	Pst 10.1-8	Pst 11.1-8	Pst 12.1-8

Supernatants from the most positive clones were re-tested after culturing for a further ten days using a RAJI cell-bound ELISA. Those clones found to secrete antibody exhibiting strong and/or perinuclear staining of RAJI cells were Pst 1.6, 2.2, 3.2, 4.2, 5.2, 6.2, 6.5, 7.7, 8.6, 9.7, 11.5, and 12.4.

Table 6.1.7 Results of JP3 and rat α-2,6-sialyltransferase ELISAs.

Pst mAb clone	Mean Absorbance at 490 nm (adjusted for n.s.b.*)			
		rat α-2,6-sialyltransferase		
	JP3	50 mU/ml	25 mU/ml	12.5 mU/ml
1.6	0.378	0.063	0.073	0.087
2.2	2.519	0.091	0.099	0.314
3.2	0	0	0	0
4.2	0.187	0.064	0.047	0.079
5.2	1.291	0.097	0.084	0.145
6.2	0.337	0	0.011	0.016
6.5	1.986	0.058	0.109	0.215
7.7	0.597	0.073	0.099	0.135
8.6	0.399	0.029	0.065	0.076
9.7	0.409	0.245	0.260	0.377
11.5	1.375	0.052	0.091	0.112
12.4	0.136	0	0.028	0.036

* n.s.b.= non-specific binding

Supernatants from 12 clones were later tested for reactivity with the JP3 peptide and with purified rat α-2,6-sialyltransferase using peptide ELISAs. Plates were coated

with JP3 or α-2,6-sialyltransferase at 50 mU/ml, 25 mU/ml and 12.5 mU/ml, and the HRP-peptide ELISA carried out as normal. The results are expressed in table 6.1.7.

Clones 2.2, 5.2, 6.5, 7.7, 9.7, and 11.5 were found to secrete high levels of JP3-specific mAb. Clones 2.2, 6.5, and 9.7 were found to secrete mAb which also appear to cross-react with rat α-2,6-sialyltransferase. All clones were cultured for a further 3 days and fresh supernatant samples were taken and isotyped using an HRP ELISA . The results of this isotyping process are given in table 6.1.8.

Table 6.1.8 **Results of Pst Isotyping.**

mAb	Absorbance at 405 nm				
	IgG1	IgG2a	IgG2b	IgG3	IgM
Pst 1.6	0.256	0.189	0.145	0.135	0.181
Pst 2.2	0.238	0.111	0.373	0.115	**2.367**
Pst 3.2	0.186	0.078	0.075	0.077	0.152
Pst 4.2	0.221	0.146	0.121	0.118	**0.435**
Pst 5.2	0.775	0.225	0.553	0.155	**2.969**
Pst 6.2	0.226	0.113	0.409	0.092	**2.425**
Pst 6.5	**2.976**	0.306	0.697	0.160	**2.457**
Pst 7.7	0.253	0.187	0.149	0.160	0.184
Pst 8.6	0.286	0.145	0.123	0.121	0.157
Pst 9.7	**OVER***	0.556	**2.459**	0.311	0.366
Pst 11.5	0.310	0.378	**0.584**	0.194	0.290
Pst 12.4	0.183	0.087	0.083	0.089	0.126
mean(-ve) + 3 s.d.	0.335	0.059	0.110	0.076	0.099

* OVER = above the limits of the plate reader i.e. ≥ 3.

Pst clones 1.6, 3.2, 7.7, 8.6, and 12.4 appeared to have stopped secreting Ab, as supernatants from these clones did not contain significant levels of any class of Ab (absorbance levels were not much greater than those of the negative controls + 3 x the standard deviation). Clones 2.2, 4.2, 5.2, and 6.2 were all found to secrete IgM. Clone 6.5 appeared to secrete a mixture of IgG1 and IgM. This clone should be identical to clone 6.2, but it is possible that it was undergoing class switch at the time of testing. Clone 9.7 appears to secrete a mixture of IgG1 and IgG2b, and clone 11.5 secretes IgG2b.

Clones 2.2, 4.2, 5.2, 6.2, 6.5, 9.7 and 11.5 were transfused into the peritoneal cavity of BALB/C mice in order to produce murine ascites, but only 5.2, 9.7 and 11.5 were successful. Pst clone supernatants and ascites were used in immunohistochemical staining of the same liver and pancreas sections previously stained for CDw75 in order to compare staining patterns. The mAb were also used to stain Western blots of CDw75 positive cell lysates, and were used in immunoprecipitations. The results of these experiments are described in section 6.2.

6.2 Molecular Weight Determination of Pst Antigen.

6.2.1 Western Blotting with Psts.

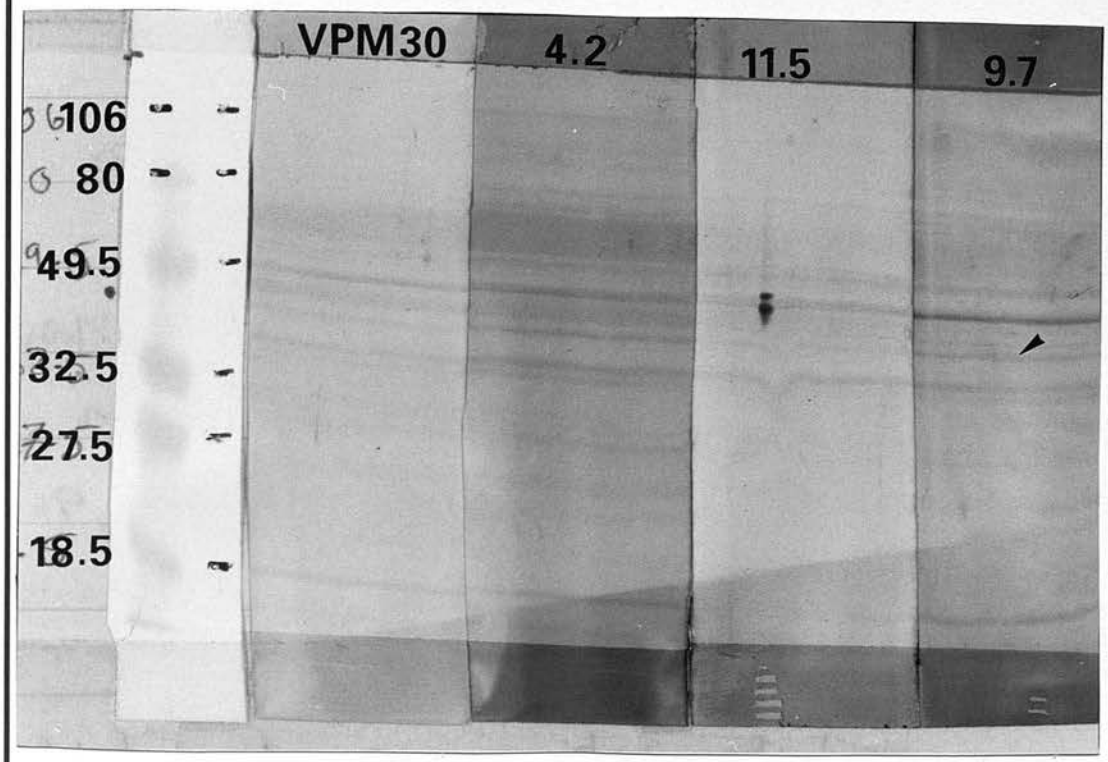
Several attempts were made to determine the molecular weights of the proteins recognised by the Pst mAbs on Western blots of electrophoresed cell lysates. The results were never clear-cut, as many non-specific bands were always stained by the control mAbs as well as the Psts. However, in every experiment, all Pst mAbs and ascites samples tested produced virtually identical protein banding patterns, staining one, and sometimes two prominent bands in each test.

An example of the results obtained from one such experiment is given in figure 6.2.1. In this experiment, PNT cells were harvested from culture, and solubilised in 0.5% CHAPS. 200μl of lysate was diluted in 800μl of reducing sample buffer and boiled at 100°C for 4 mins. 400μl aliquots of this were loaded on to two single sample-well SDS (10% Polyacrylamide) gels with rainbow molecular weight markers in the marker lanes. Electrophoresis was carried out, and separated proteins transferred to nitro-cellulose by Western blotting. After blocking unbound sites by incubating in PBS 5% dried milk powder overnight at 4°C, blots were washed and cut into strips. Individual strips were incubated in Pst culture supernatant or ascites diluted 1/1000 in PBS 0.5% Tween. Antibodies used are listed in table 6.2.1.

Antibody-treated strips were stained by enzyme labelling with HRP and developed using DAB. Stained strips were reassembled and are shown in figure 6.2.1. Two specific major bands are visible on all Pst mAb-labelled strips. The positions of these specific protein bands were measured, and their molecular weights calculated using the formula of the standard curve generated by the molecular weight markers as illustrated in figure 6.2.2.

Figure 6.2.1 Western Blots Labelled with Pst mAbs

Gel 1



Gel 2

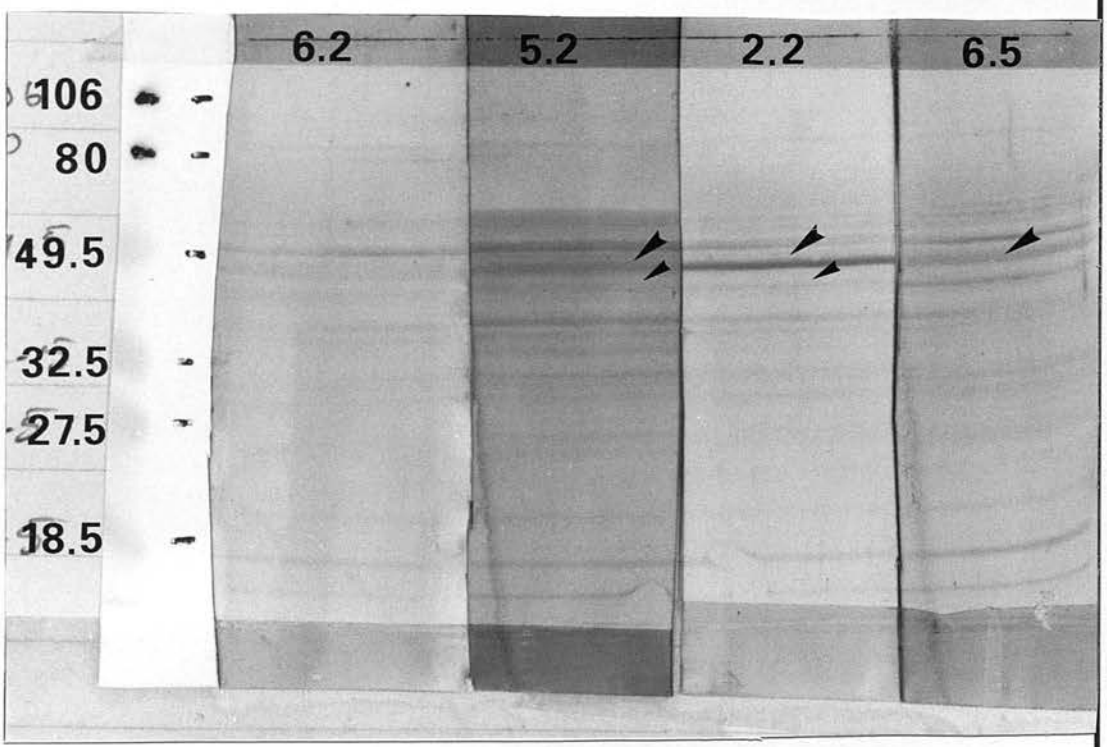
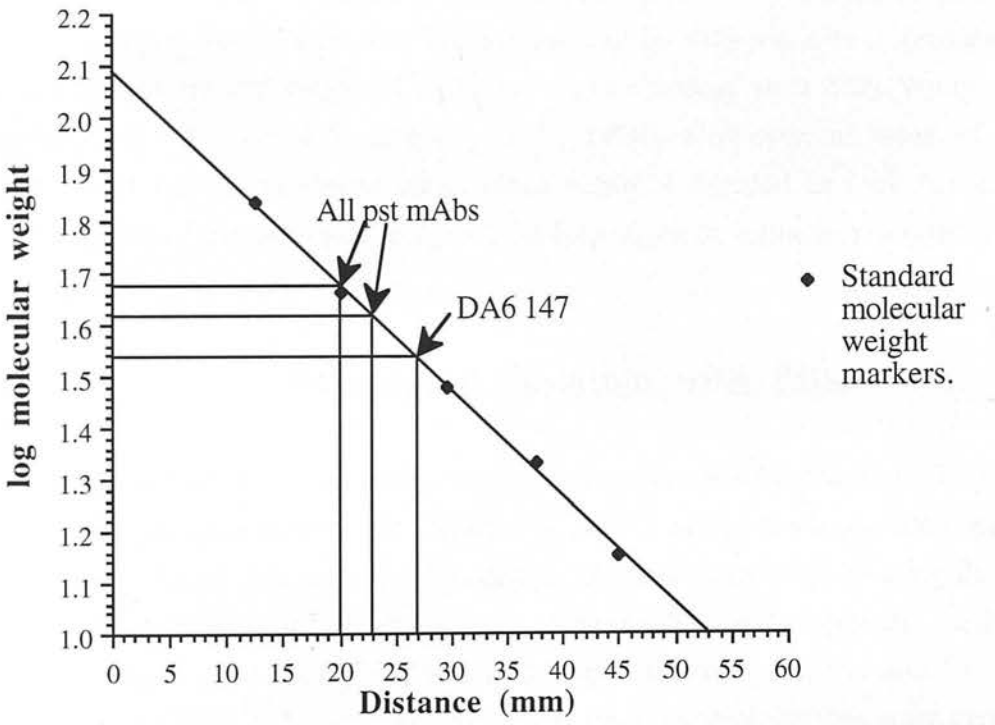


Table 6.2.1 **mAbs used to label Western blot shown in figure 6.2.1**

GEL 1	GEL 2
negative control VPM30	Pst 6.2 supernatant
Pst 4.2 supernatant	Pst 5.2 supernatant
Pst 11.5 supernatant	Pst 2.2 supernatant
Pst 9.7 supernatant	Pst 6.5 supernatant

Figure 6.2.2 **Standard curve for blots in Fig 6.2.1**
y = 2.0877 - 0.020571x R^2 = 0.998



The positions of the two specific bands detected (not found with control mAbs) are illustrated in figure 6.2.2. The molecular weights of these proteins were calculated using the formula of the curve, and are listed in table 6.2.2 below.

Table 6.2.2 Molecular weights of proteins detected in Fig 6.2.1.

mAb	Band positions (mm)	log (molecular weights)	Molecular weights (kD)
Pst 2.2, 5.2, 6.5	20	1.68	47.5
Pst 2.2, 5.2, 9.7	23	1.61	41.2

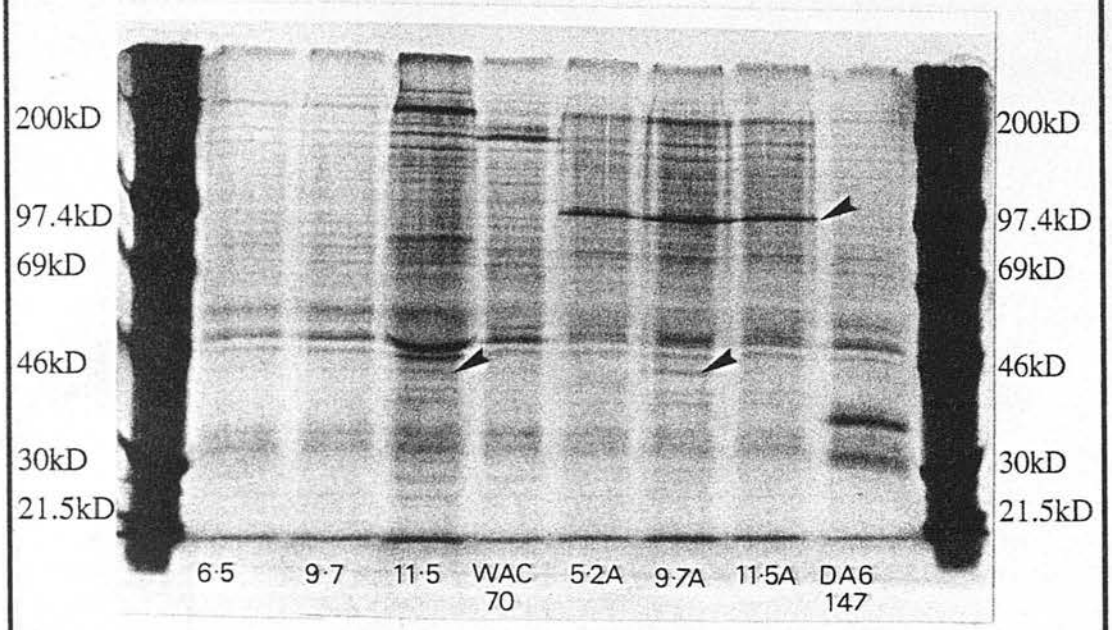
The predicted molecular weight of the protein component of β-galactoside-α-2,6-sialyltransferase is 46.367 kD(Stamenkovic, I., Asheim, H.C., Deggerdal, A., Blomhoff, H.K., Smeland, E.B. and Funderud, S., 1990). As the bands detected by the Pst mAbs are of molecular weights 47.5 and 41.2 kD, it is very possible that these mAbs do in fact recognise different forms of this sialyltransferase enzyme, especially since it is known that in animals, the enzyme can be differentially expressed in different tissues by utilisation of different transcriptional start sites(Wang, X., O'Hanlon, T.P., Young, R.F. and Lau, J.T., 1990). However, as none of the Western blots stained produced clear, clean bands, I decided to look for more conclusive proof of the molecular weight of the Pst antigen by immunoprecipitation.

6.2.2 Immunoprecipitation with Psts.

PNT cells were metabolically labelled with ^{35}S-met + cys, and solubilised in 1% Triton CF-54 (it was not necessary to use CHAPS as the Pst epitopes should not contain carbohydrate). After several unsuccessful attempts at immunoprecipitation using Biorad immunobeads, precipitations were carried out using the Protein-A-Sepharose method. Beads were armed using Pst 6.5, 9.7 and 11.5 culture supernatants, and also Pst 5.2, 9.7, and 11.5 and DA6 147 and WAC 70 ascites. Immunoprecipitations were carried out, and precipitates were run on an SDS 10% Polyacrylamide gel. The gel was Western blotted and exposed to film for several days before developing, and is shown in figure 6.2.3.

A single major band was detected in the ascites immunoprecipitates only, and another weaker, but specific band was seen in lanes 4 & 7 (Pst 11.5 supernatant and Pst 9.7 ascites precipitates). The positions of these bands were measured, and their molecular weights calculated using the formula of the standard curve generated by the molecular weight markers, as shown in figure 6.2.4. The values obtained are listed in table 6.2.3.

Figure 6.2.3 Western blot of Pst Immunoprecipitates.



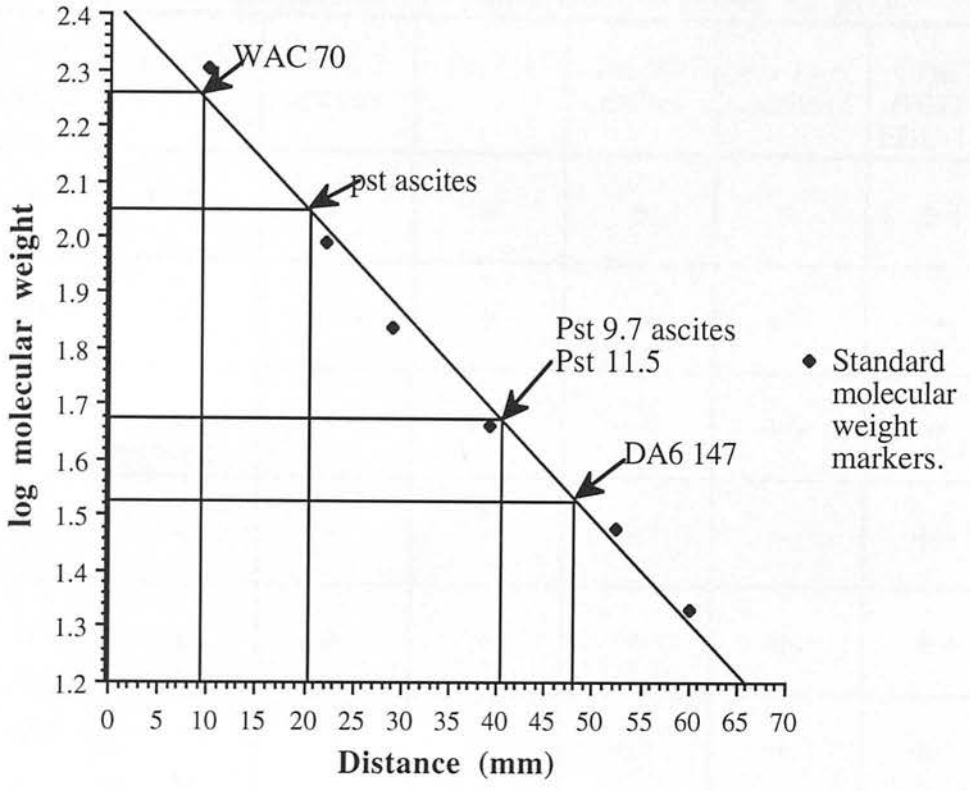
Arrows indicate Pst-specific bands at 112.4 kD and at 46.8 kD.

Table 6.2.3 Molecular weights (m.w.) of Pst Immunoprecipitates.

mAb	Distance on gel	log m.w.	m.w.
WAC70(CD11a)	9.5 mm	2.254	179.6
DA6 147 (MHC II)	47.5 mm	1.533	34.1
Pst 11.5	41 mm	1.67	46.8
Pst 9.7 Ascites	41 mm	1.67	46.8
Pst 5.2 Ascites	20.5 mm	2.05	112.2
Pst 9.7 Ascites	20.5 mm	2.05	112.2
Pst 11.5 Ascites	20.5 mm	2.05	112.2

The molecular weights obtained for the CD11a and MHC class II antigens are correct, and the predicted molecular weight of human β-galactoside-α-2,6-sialyltransferase is around 46 kDa. It is therefore possible that the 46.8 kDa band precipitated by Pst mAbs 11.5 and 9.7 is in fact human α-2,6-sialyltransferase. It is also possible that the 112 kDa band detected may be a dimer of the sialyltransferase enzyme as it is known that in rats the enzyme probably operates as a dimer(Fleischer, B., McIntyre, J.O. and Kempner, E.S., 1993).

Figure 6.2.4 Standard curve for blot in Fig 6.2.3
y = 2.43 - 0.0185x R^2 = 0.985



6.3 Tissue Distribution of Pst Antigen.

The same tissue samples of liver and pancreas as studied in chapter 4 were stained using immunohistochemical techniques in order to compare distribution of the Pst antigen(s) with that of CDw75, and to compare expression levels on normal and tumour tissue. As in chapter 4, the number and quality of the tissue samples available were limited, and results can only be interpreted as a preview to more exhaustive research.

6.3.1 Liver Sections

Ten of the same samples of liver tissue as used in chapter 4, were immunohistochemically stained with five clones of Pst mAbs. The results are indicated in table 6.3.1 below. Examples of some of the positively stained tissue samples are also shown in photographic plates 6.3.1 - 6.3.3. Texas red was used as the

developing substrate in these sections, and positive staining is therefore indicated by various shades of pink colouring.

Table 6.3.1 Results of Pst and CDw75 staining on liver.

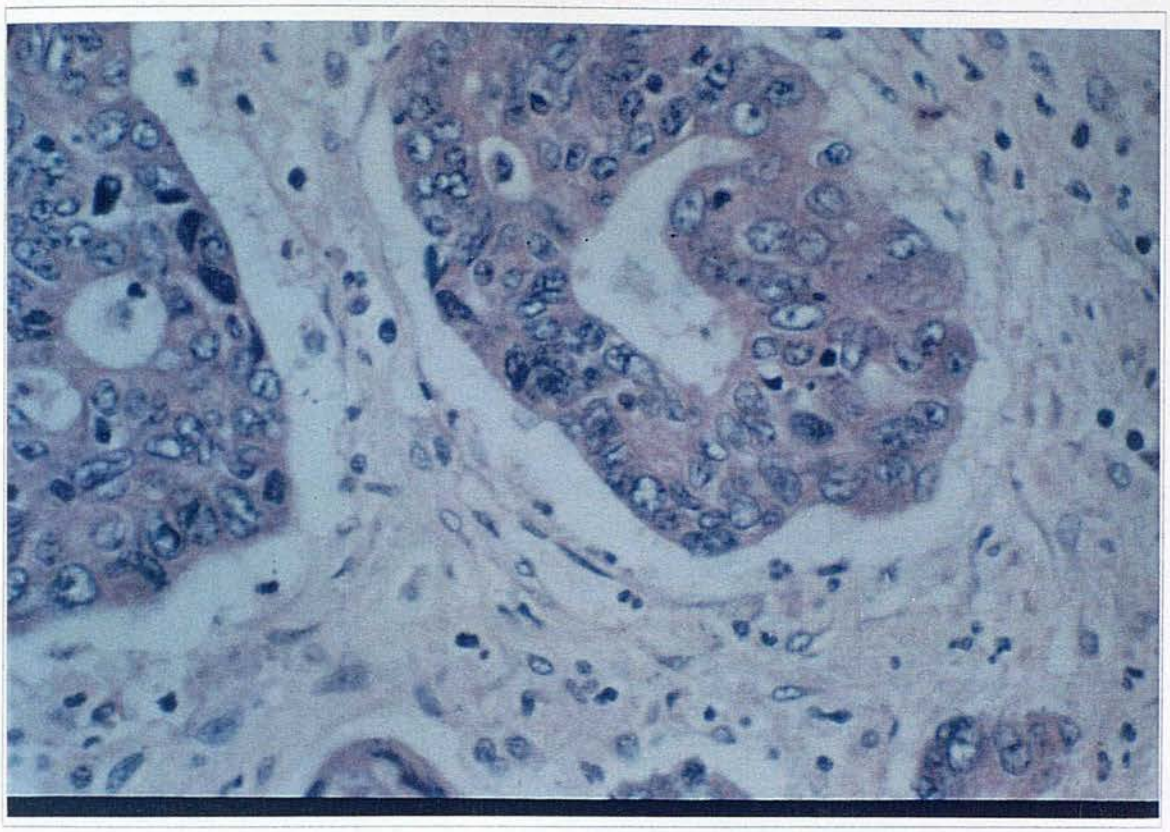
Tissue Sample	Pst 4.2	Pst 5.2 ascites	Pst 6.5	Pst 9.7 ascites	Pst 11.5 ascites	CDw75 (HH2 & EBU-141)
1	N.D.	+	+	+	+	++
1(t)*	-	-	+	+	+/-	-
2	-	-	+/-	+/-	+/-	+
2(t)	-	-	-	-	-	+/-
3	+	+	+	+	+	++
3(t)	-	-	+/-	+/-	+	+/-
4	-	+/-	-	-	-	++
4(t)	-	-	-	-	-	+/-
5	+/-	+	++	+/-	+	+
5(t)	+/-	+/-	++	-	+/-	+/-

* (t) indicates a sample of tumour tissue taken from the same patient.

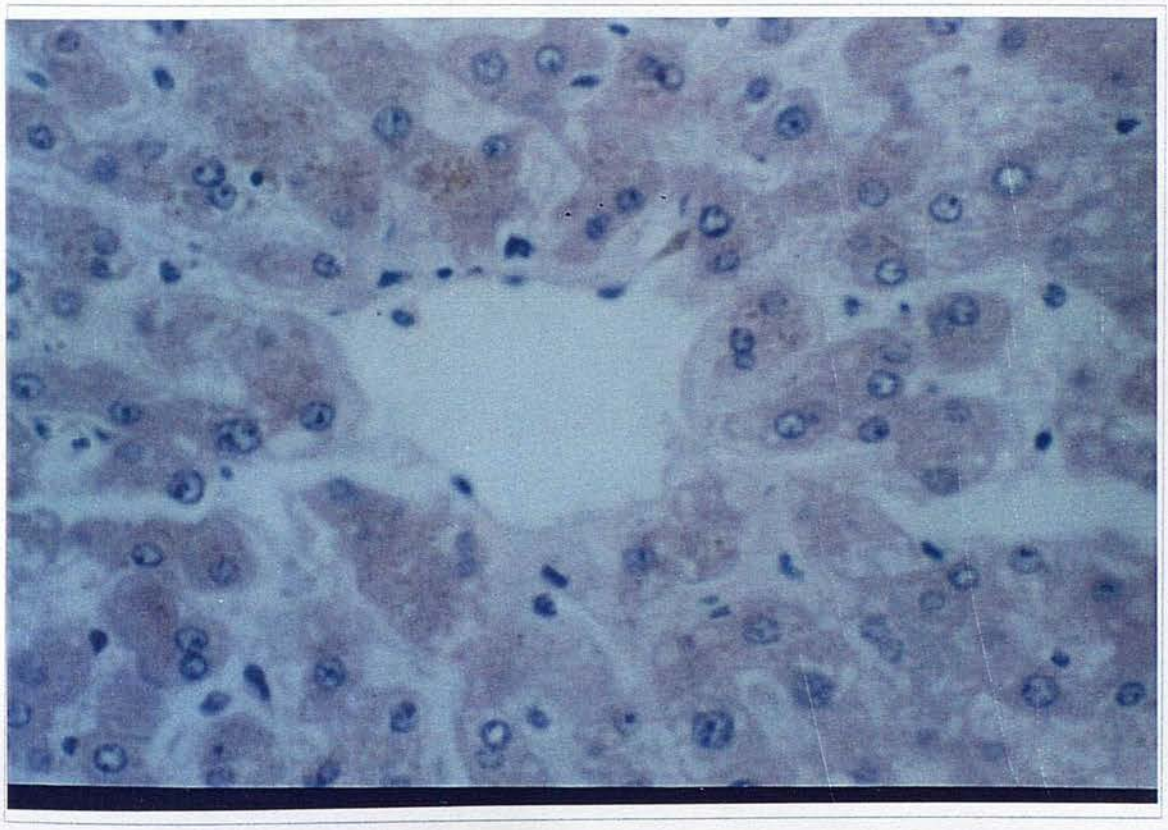
+/- indicates very weak staining, + indicates moderate staining,

++ indicates strong staining, - indicates no staining.

Photographic Plate 6.3.1

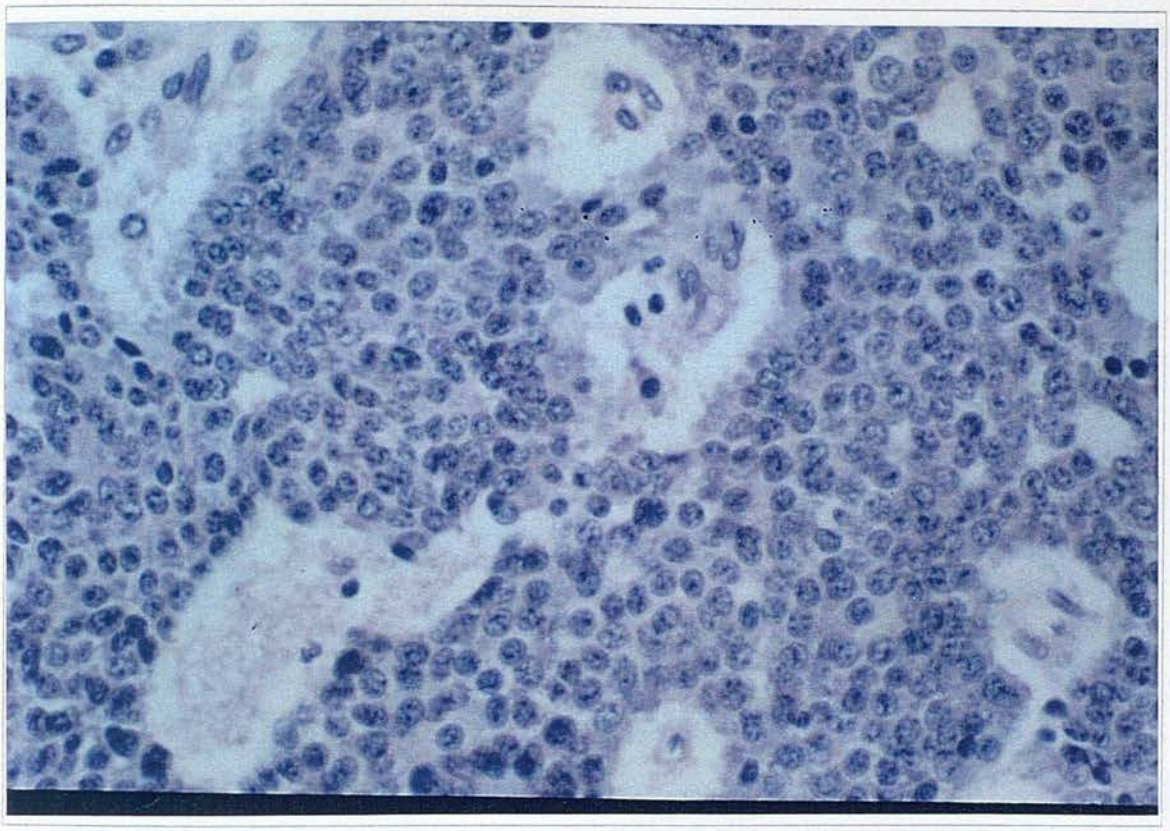


(a) Pst 9.7 staining of liver 1(tumour). (x 20 magnification).

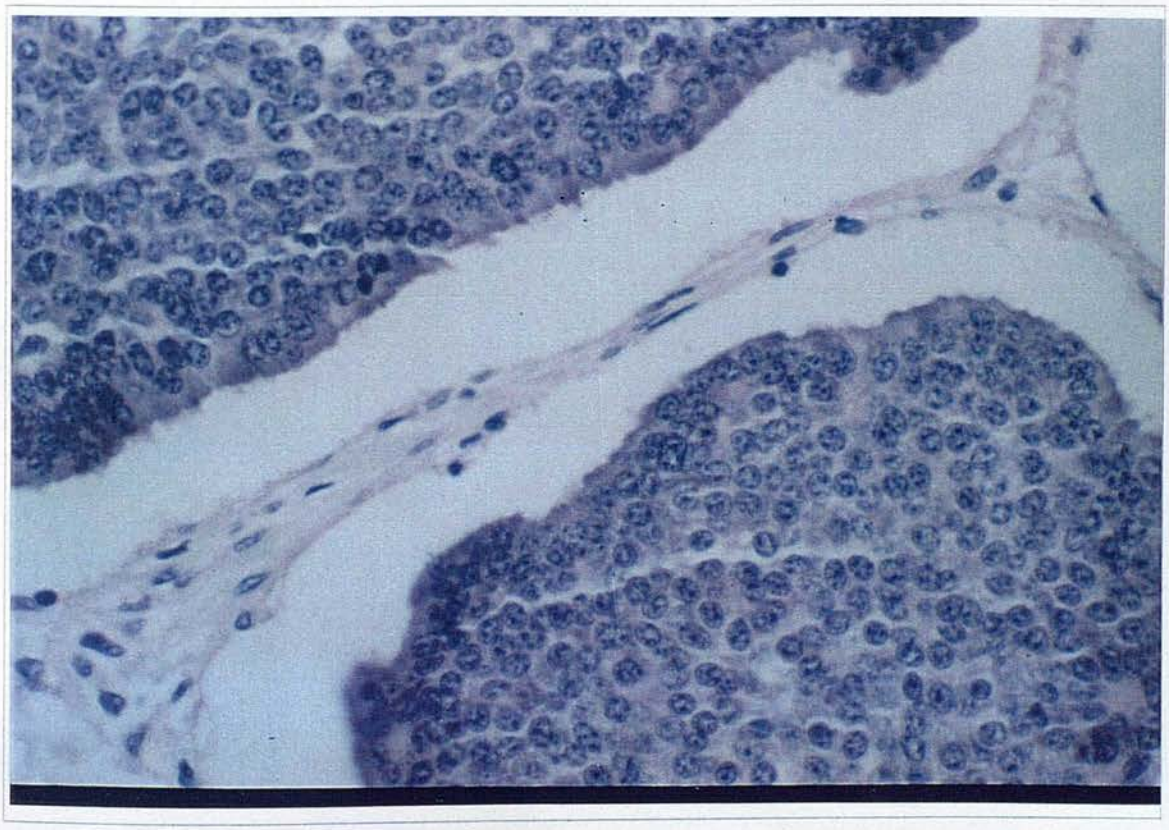


(b) Pst 6.5 staining of liver 3 (normal). (x 40 magnification).

Photographic Plate 6.3.2

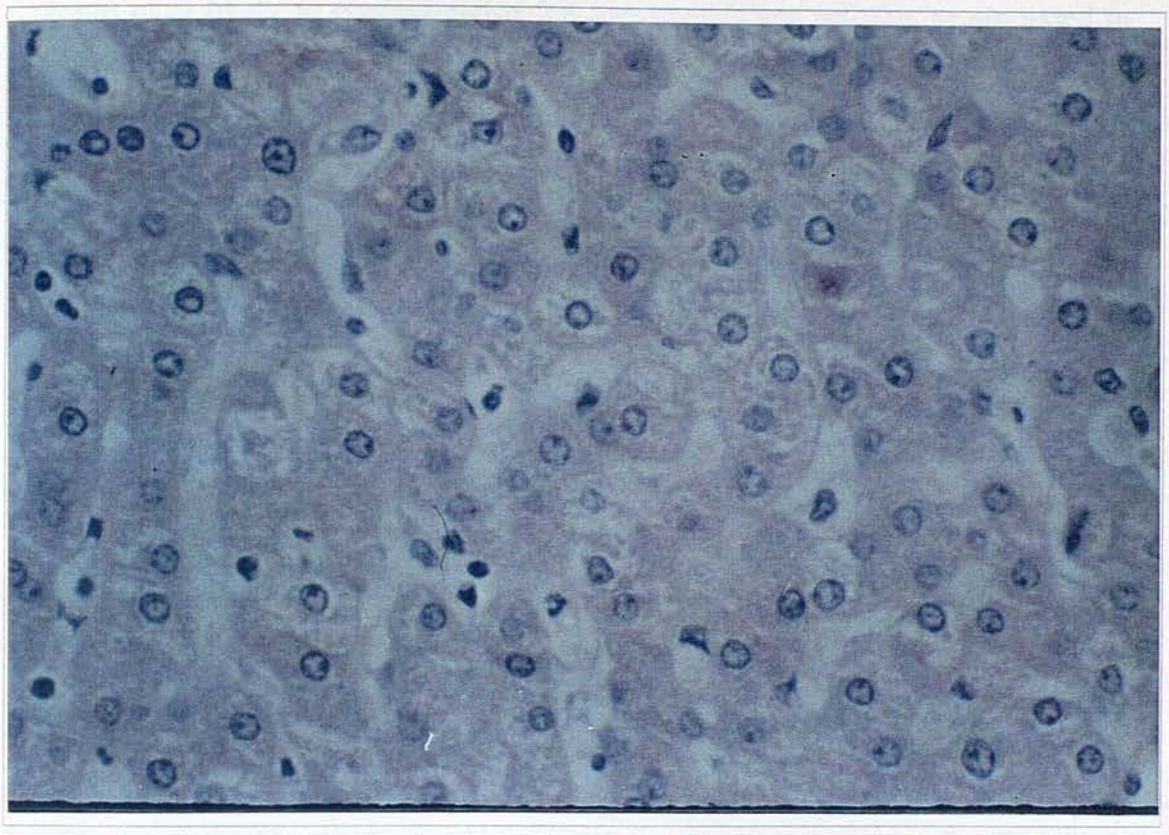


(a) Pst 6.5 staining of liver 3 (tumour). (x 10 magnification)

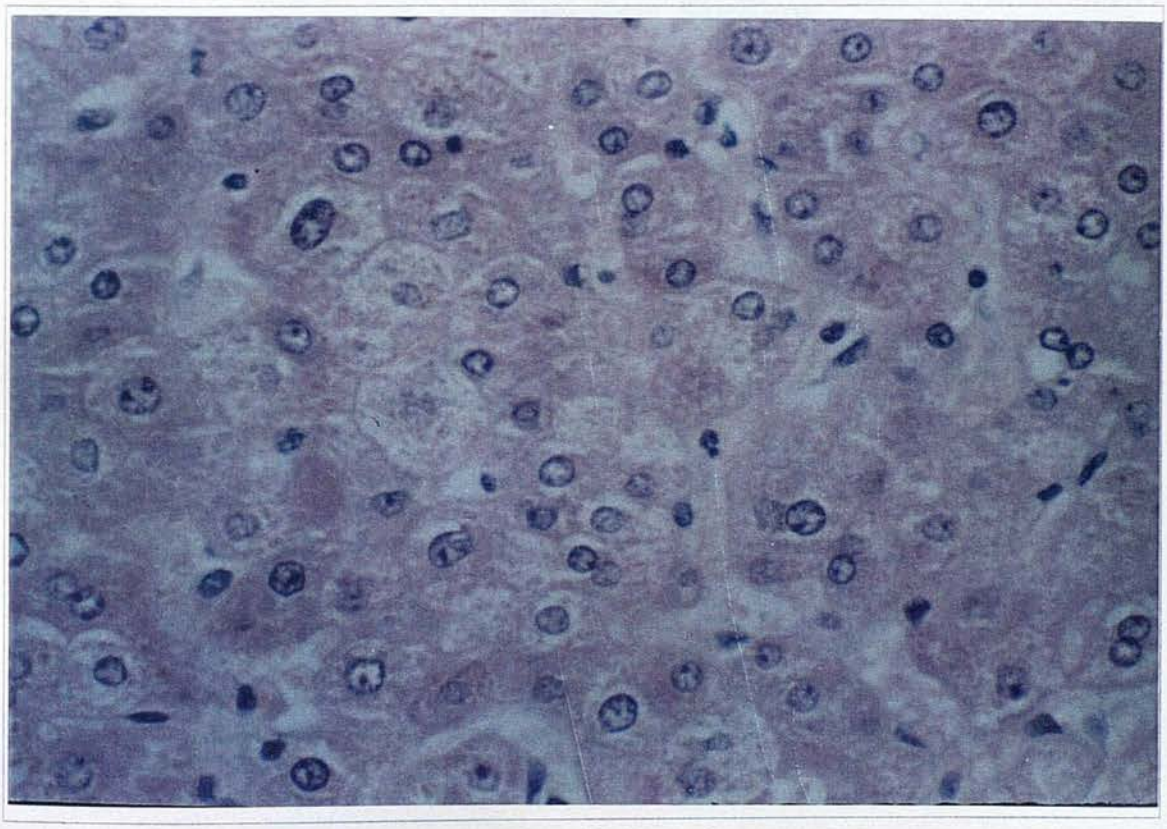


(b) Pst 9.7 asc. staining of liver 3 (tumour). (x 10 magnification)

Photographic Plate 6.3.3



(a) Pst 5.2 asc. staining of liver 5 (normal). (x 40 magnification)



(b) Pst 6.5 staining of liver 5 (normal). (x 40 magnification)

The results of this study indicate that there may be a reduction in expression of the antigen(s) recognised by Psts 5.2, 9.7 and 11.5 in tumour cells. These mAbs are the clones most likely to possess anti-α-2,6-sialyltransferase activity, as discussed in sections 6.2.1 and 6.2.2. It is possible therefore that expression of this enzyme is reduced in tumour cells, as is that of CDw75. However, due to the limited number of samples used in this study, no significant conclusions may be drawn, and a much larger group of patient samples would be required in order to fully determine any significant difference in expression levels. In addition, the identity of the antigen(s) recognised by each mAb would need to be conclusively proved.

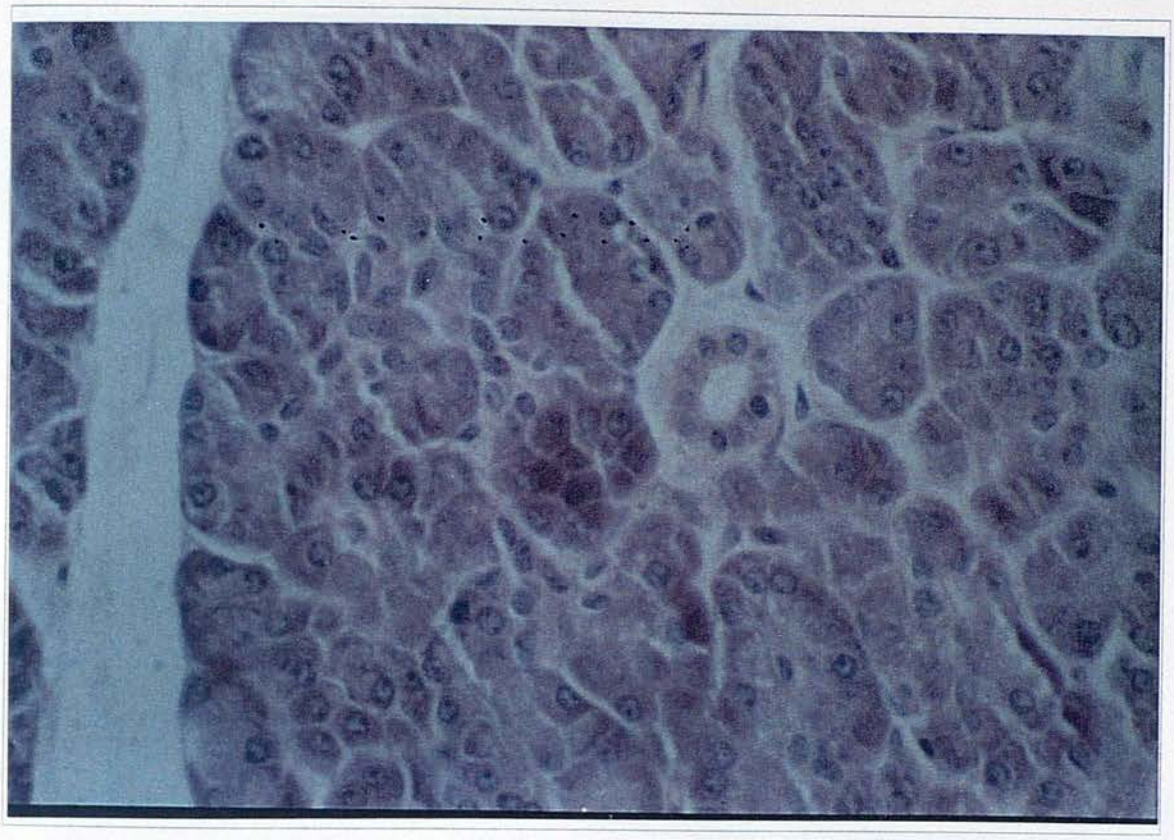
It can also be seen from photographic plates 6.3.1 - 6.3.3 that the staining pattern produced by the Pst mAbs on liver is completely different to that produced by the CDw75 mAbs. If we compare stained sections of liver 3 as illustrated on photographic plates 4.5.1 and 4.5.3 with those on photographic plates 6.3.1 and 6.3.2 the cellular distribution of the two antigens is completely different. Whereas the CDw75 mAbs predominantly stain the liver canaliculae, the Pst mAbs produce a granular cytoplasmic staining pattern which is perinuclear in location when viewed at higher magnification (6.3.1b & 6.3.3a & b). Since the sialyltransferase enzyme is found mainly in the Golgi apparatus, the staining patterns produced give further evidence that the Pst mAbs may indeed be specific for this enzyme.

6.3.2 Pancreas Sections

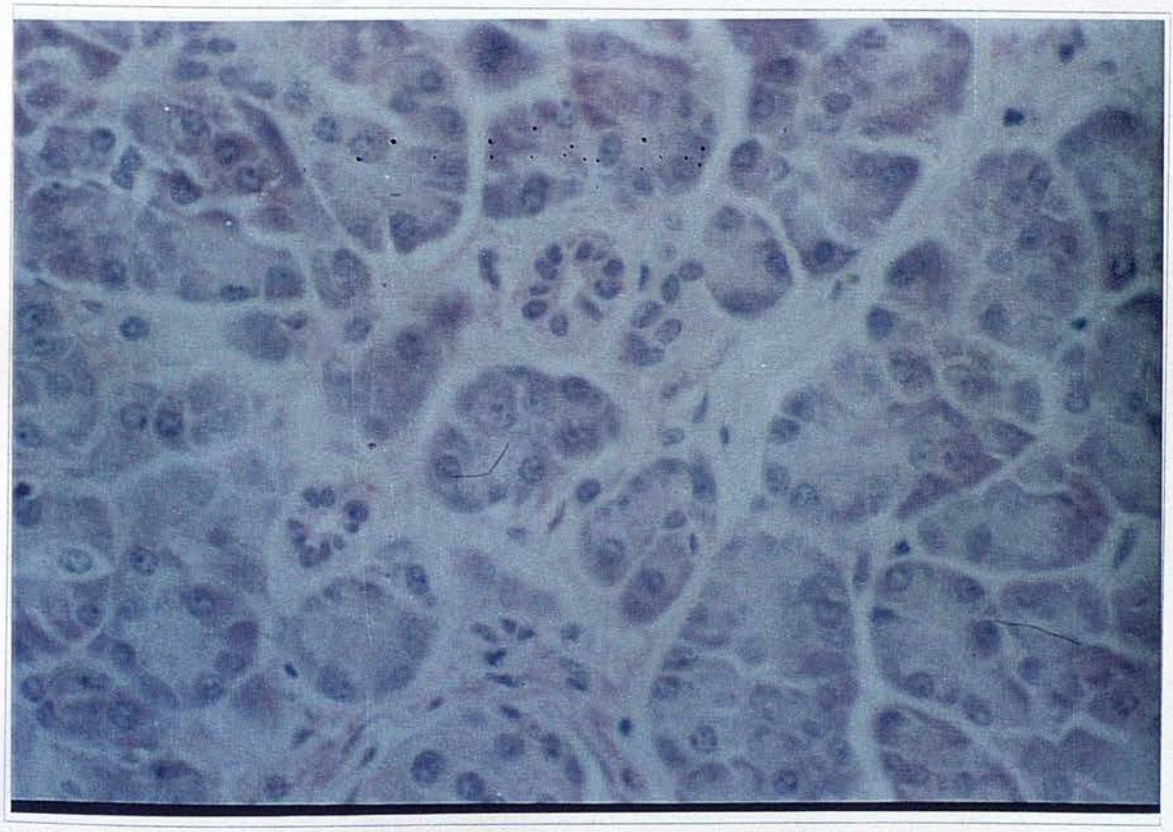
Four samples of normal pancreas and three samples of tumour pancreas were immunohistochemically stained with five Pst mAbs. The results are indicated in table 6.3.2, and examples of positively stained sections are shown on photographic plates 6.3.4 - 6.3.8.

If CDw75 and Pst mAb staining of normal pancreas sections are compared (photographic plates 4.6.1 and 6.3.4 - 6.3.7 respectively), differences in expression patterns can again be seen. CDw75 mAbs in general produce a patchy staining pattern mainly on the cytoplasm of ductal cells, but also on the membranes of these cells. Pst staining in contrast is more uniformly distributed across the section, and as in liver samples tends to stain cytoplasmic granules. However, in tumour tissue, the staining pattern produced by both sets of mAbs is very similar (photographic plates 4.6.2 and 6.3.8). With the Pst mAbs staining is much less uniform than in normal tissue, and

Photographic Plate 6.3.4

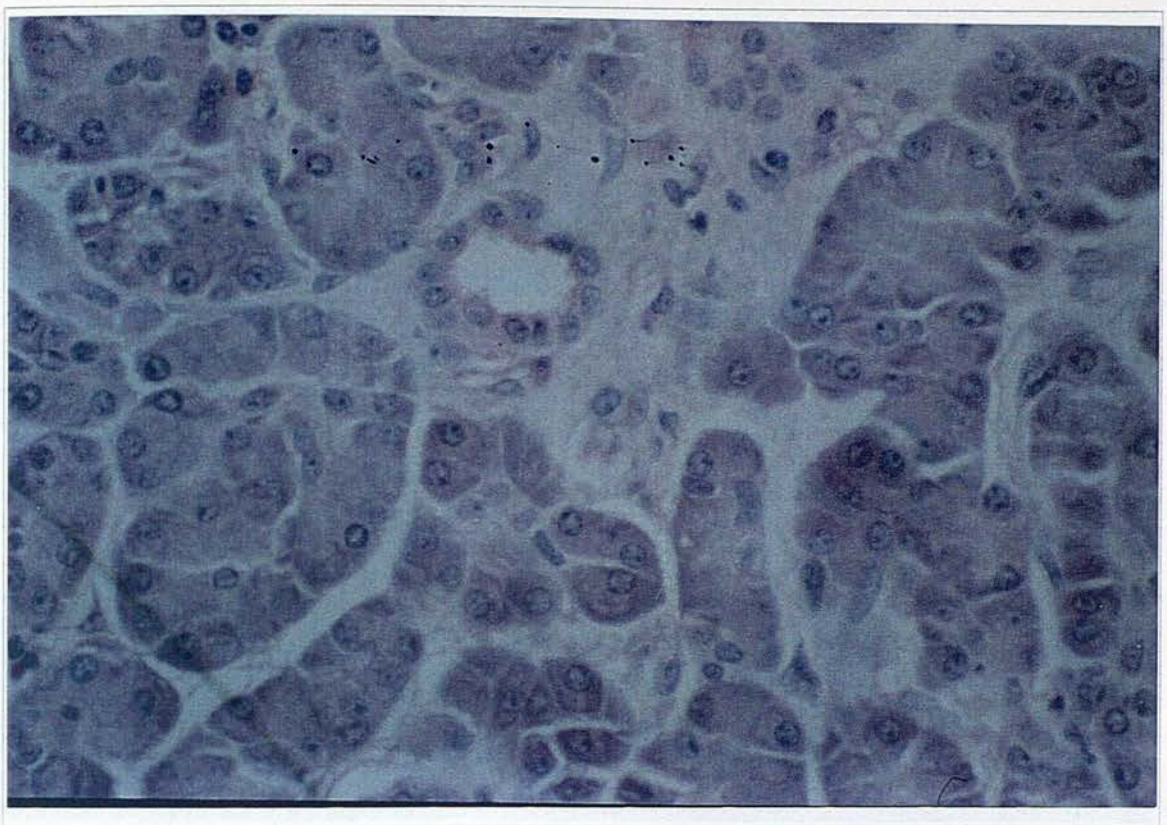


(a) Pst 6.5 staining of pancreas 2 (normal). (x 20 magnification)

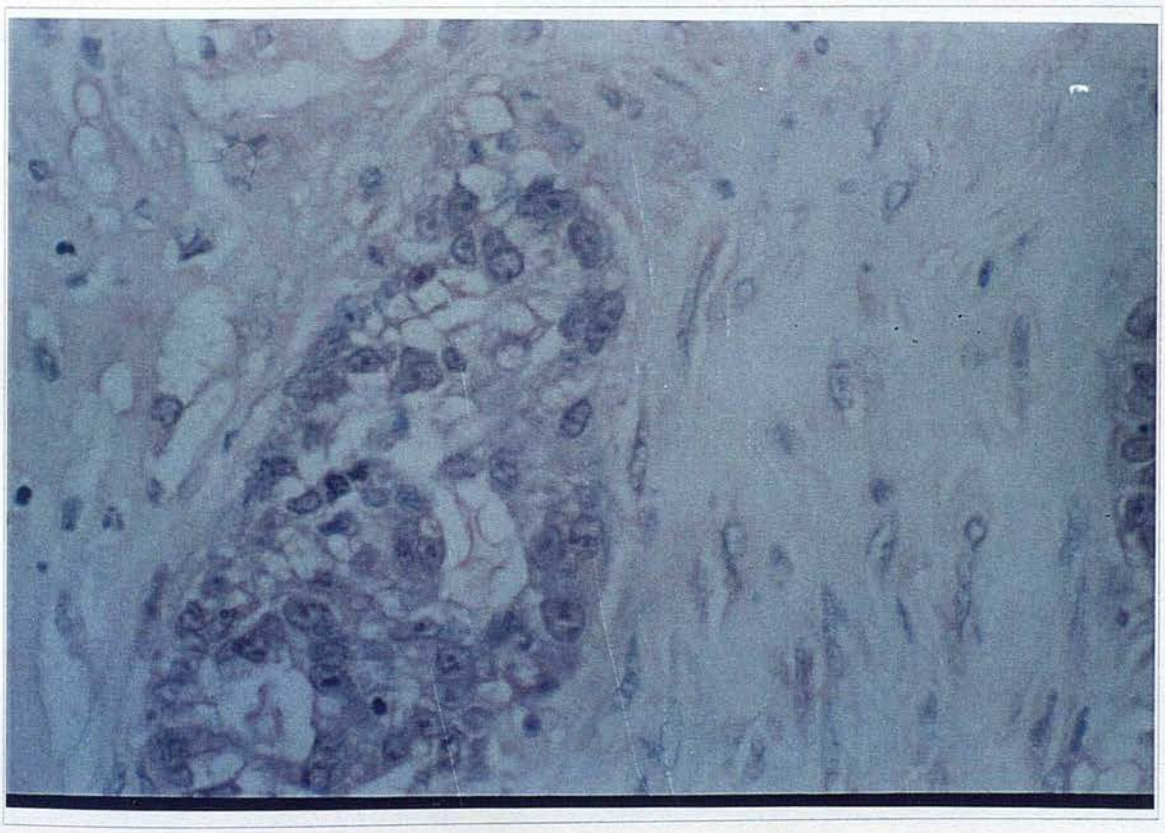


(b) Pst 9.7 staining of pancreas 2 (normal). (x 40 magnification)

Photographic Plate 6.3.5

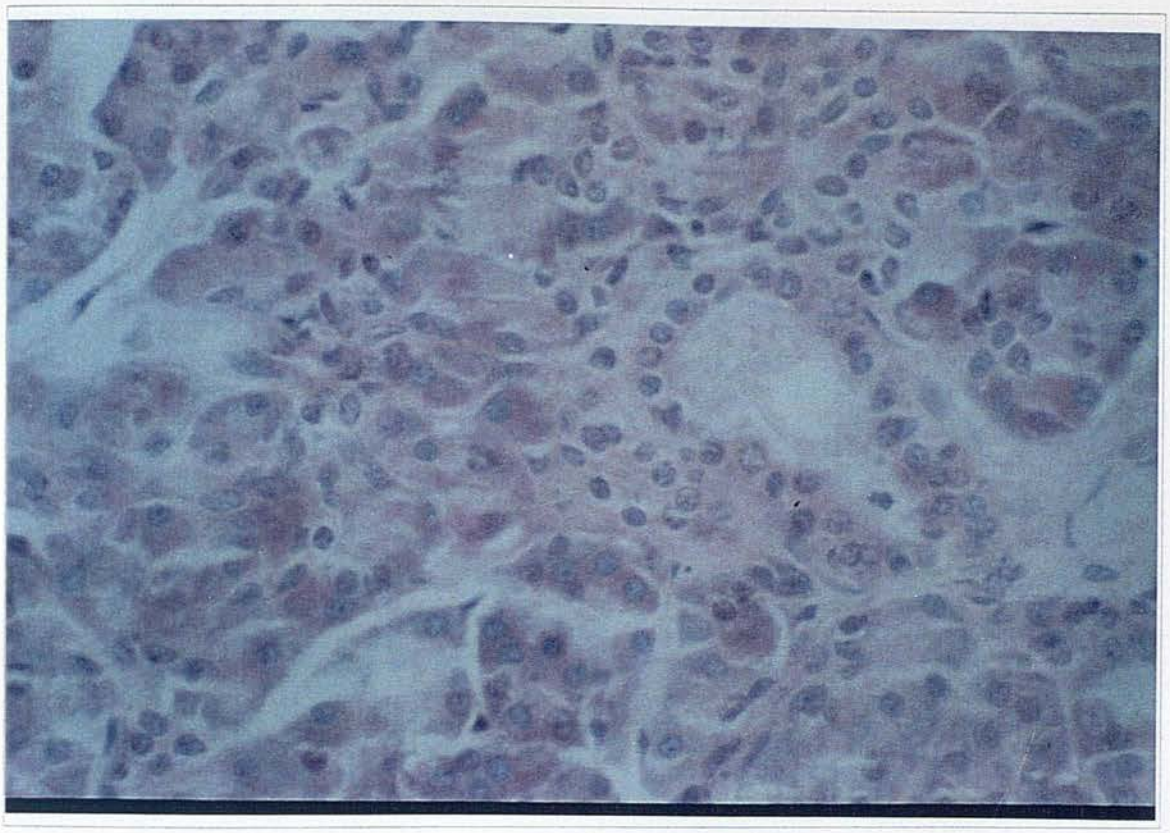


(a) Pst 11.5 staining of pancreas 2 (normal). (x 40 magnification)

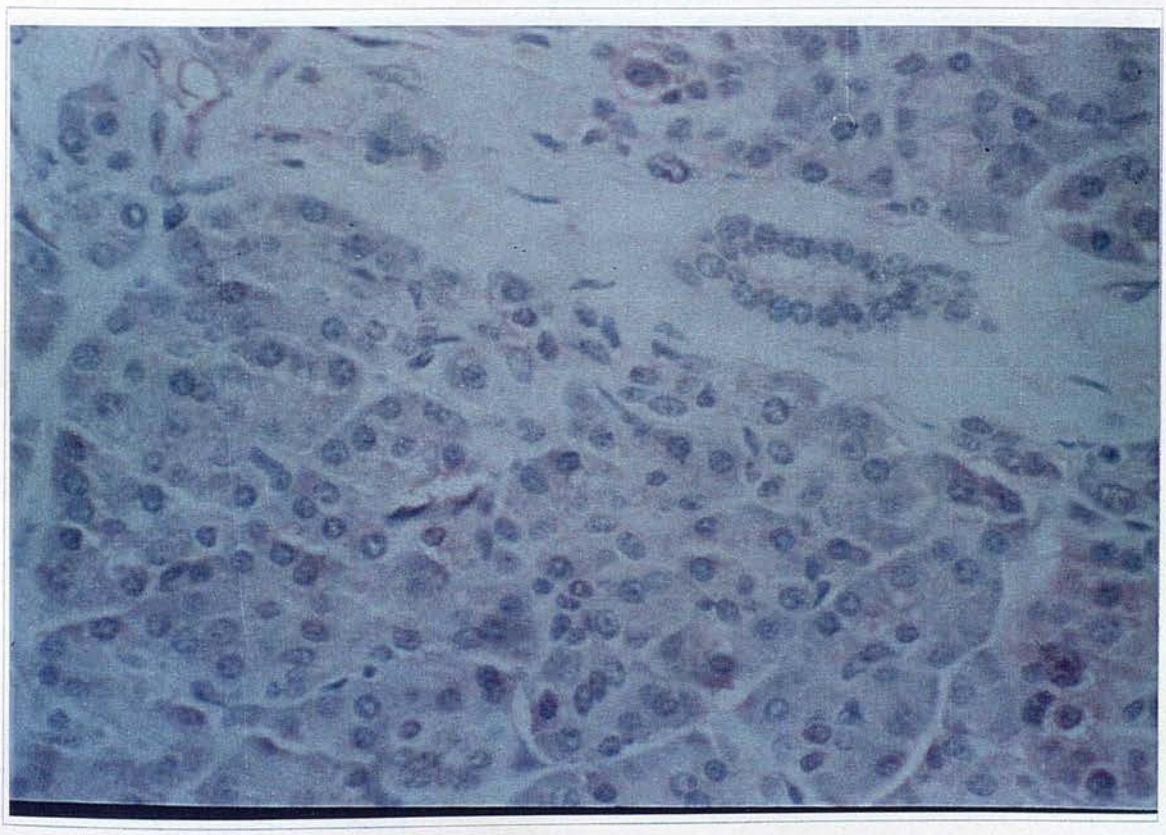


(b) Pst 11.5 staining of pancreas 2 (tumour). (x 40 magnification)

Photographic Plate 6.3.6

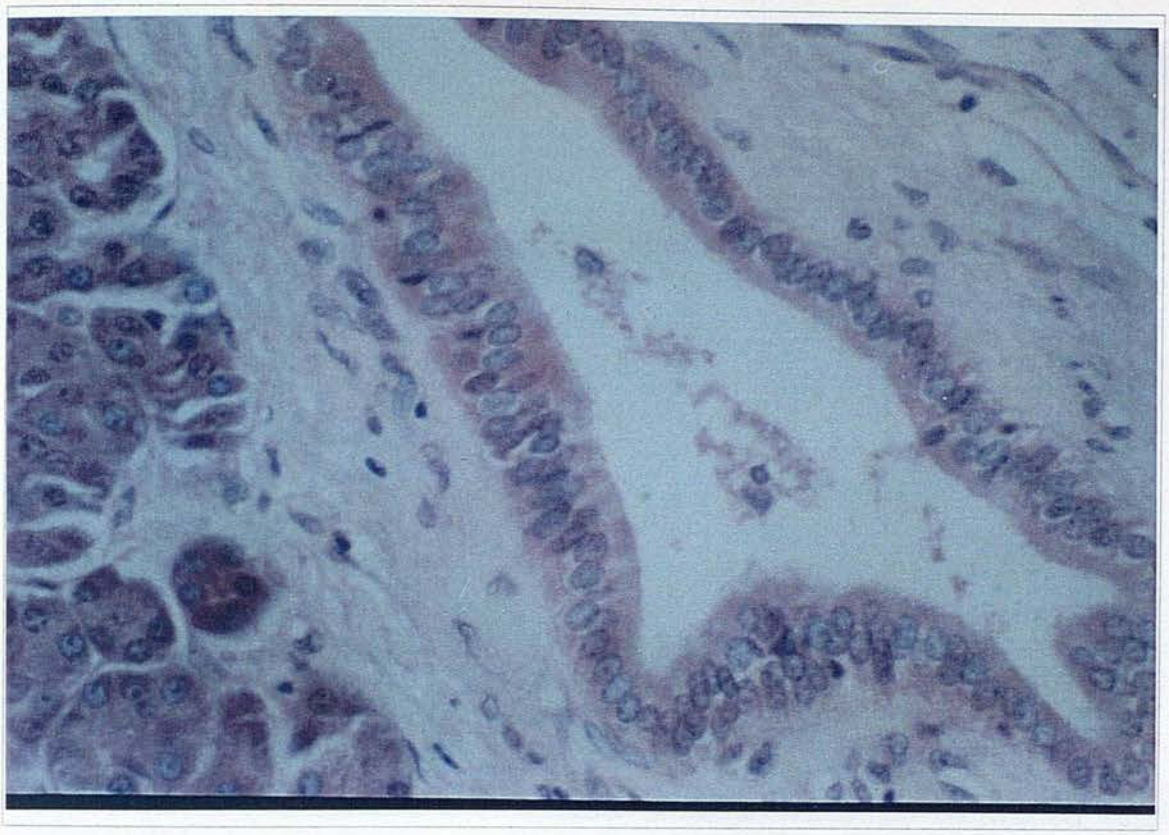


(a) Pst 6.5 staining of pancreas 5 (normal). (x 20 magnification)

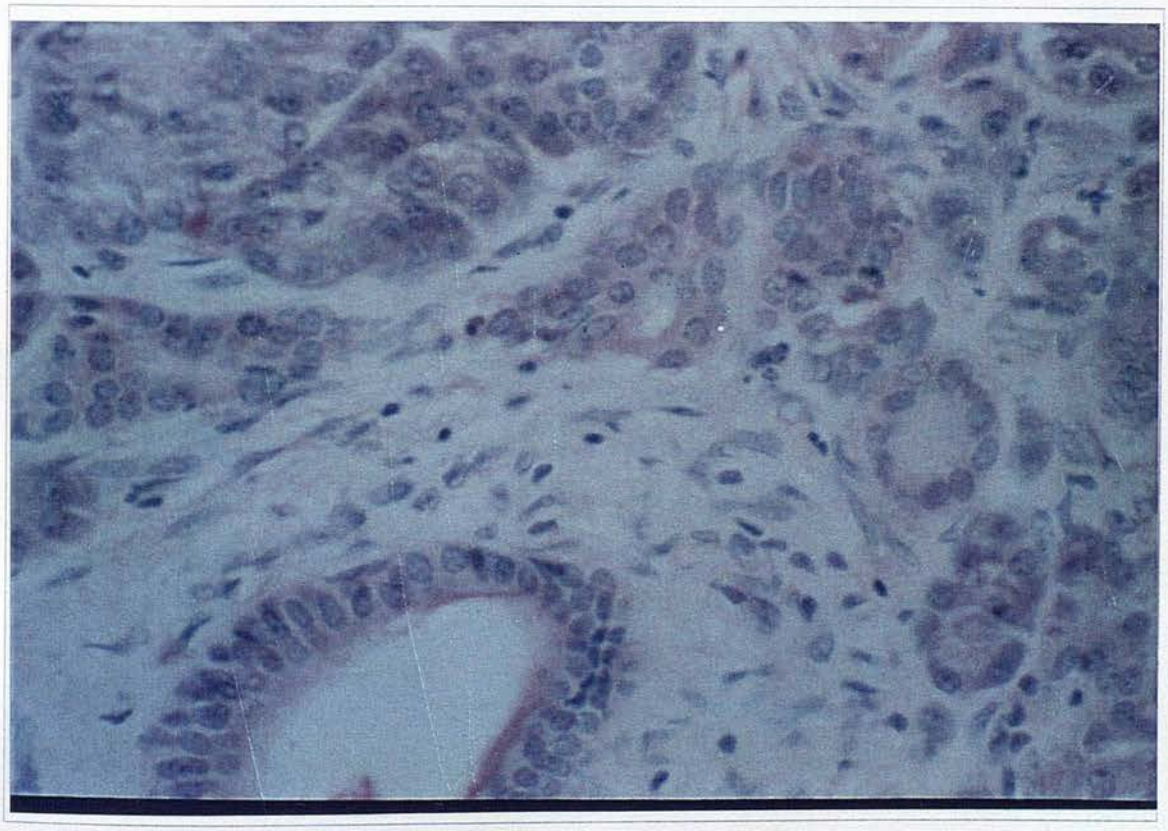


(b) Pst 11.5 staining of pancreas 5 (normal). (x 20 magnification)

Photographic Plate 6.3.7

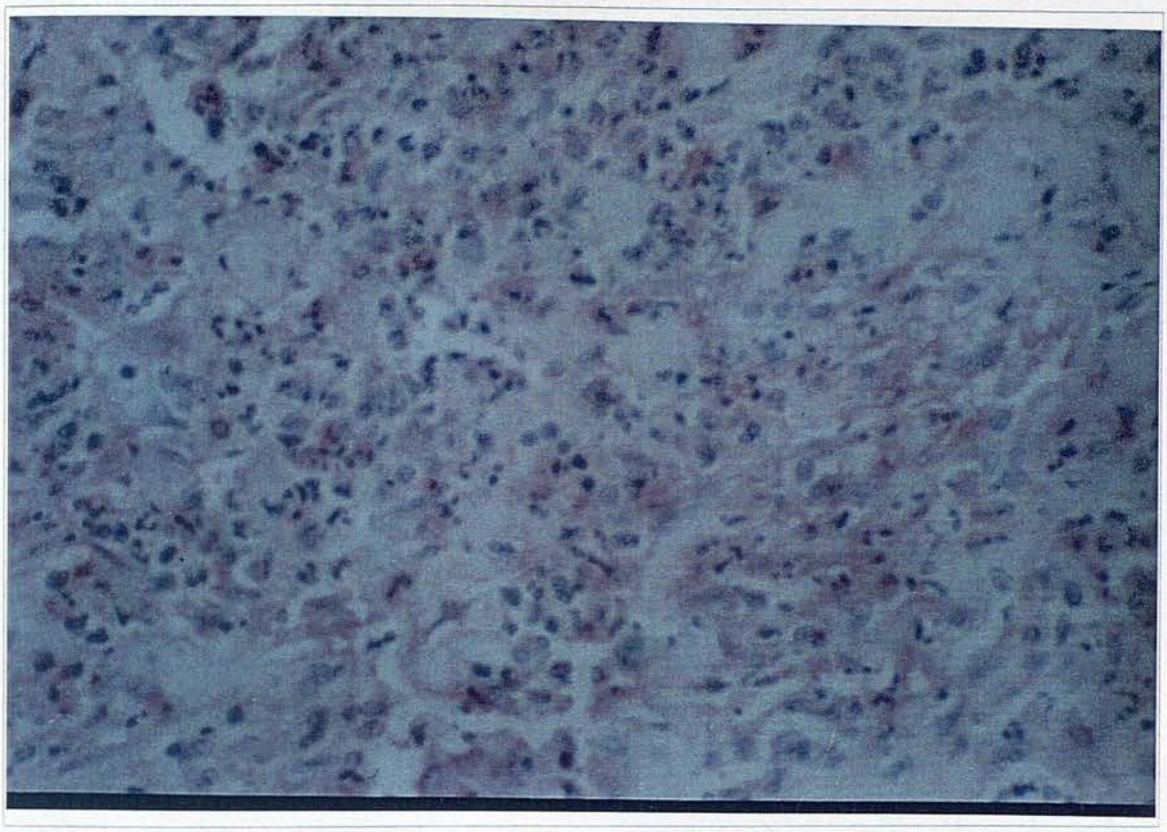


(a) Pst 6.5 staining of pancreas 4 (normal). (x 40 magnification)

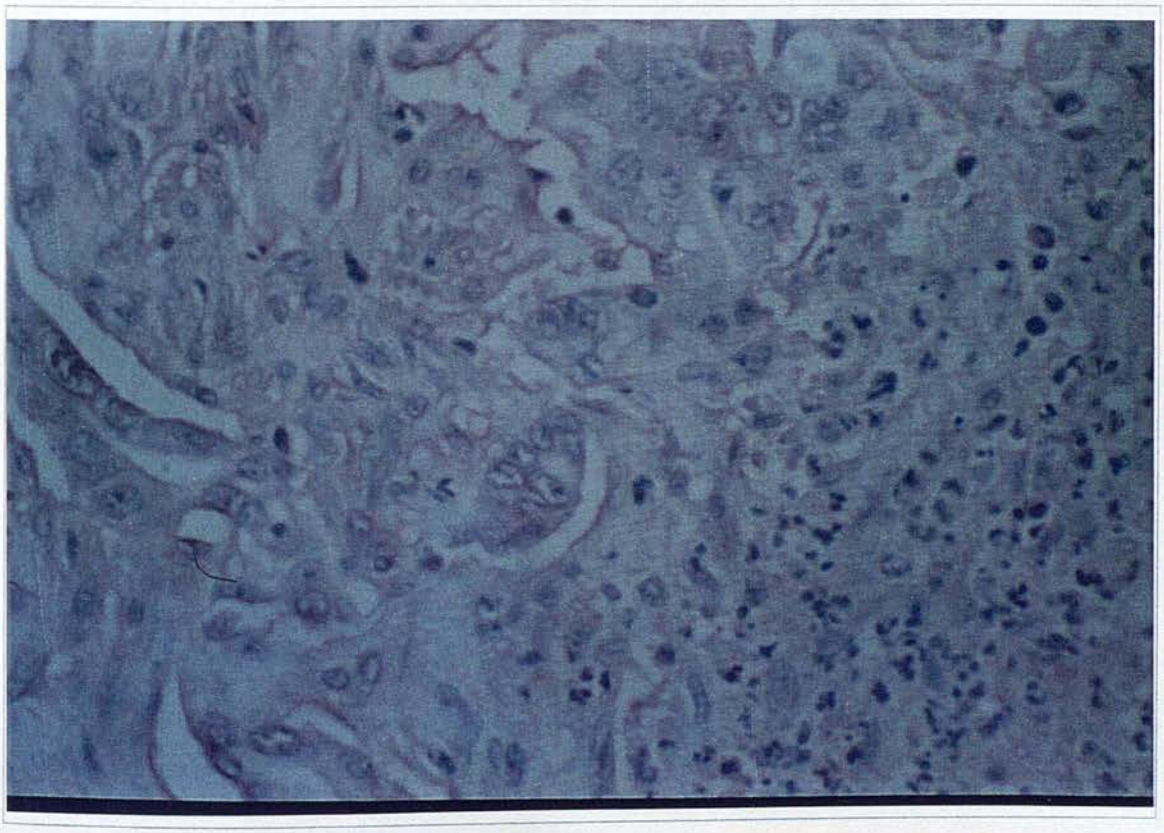


(b) Pst 11.5 staining of pancreas 4 (normal). (x 40 magnification)

Photographic Plate 6.3.8



(a) Pst 9.7 staining of pancreas 4 (tumour). (x 10 magnification)



(b) Pst 11.5 staining of pancreas 4 (tumour). (x 10 magnification)

membranous structures are also stained, indicating a total disruption of the Golgi-targeting mechanism of the sialyltransferase, and possibly production of a soluble form or a plasma-membrane-bound form of the sialyltransferase enzyme.

Table 6.3.2 Results of Pst and CDw75 staining on pancreas.

Tissue Sample	Pst 4.2	Pst 5.2A	Pst 6.5	Pst 9.7A	Pst 11.5A	CDw75 (HH2 & EBU-141)
1	-	-	-	-	-	+/-
1(t)*	-	-	-	-	-	+/-
2	+/-	+/-	+	+	+/-	+/-
2(t)	-	-	+/-	-	+/-	+/-
4	-	++	++	-	+	+/-
4(t)	+/-	+/-	+/-	++	++	+/-
5	+/-	+/-	+	+/-	+	+/-

* (t) indicates a sample of tumour tissue taken from the same patient.
+/- indicates very weak staining, + indicates moderate staining,
++ indicates strong staining, - indicates no staining.

It is unfortunate that these results are less conclusive than those on liver sections since they are more difficult to quantify, and a much larger specimen group would have to be studied in order to determine any significant and consistent differences in CDw75 or Pst mAb staining patterns between normal and tumour tissues.

6.4 Discussion.

The results of the experiments described in this chapter indicate that at least some of the mAbs produced from the synthetic peptide MAPs may be specific for the elusive β-galactoside-α-2,6-sialyltransferase enzyme. The initial screening process by Raji cell-bound ELISA selected mAbs exhibiting perinuclear staining, and this was also

observed on immunohistochemical staining of liver and pancreas sections. In addition, all Pst mAbs immunolabelled protein bands of m_r ~47 kDa and 41 kDa on electrophoretically separated preparations of detergent solubilised PNT cells. Also, Psts 11.5 and 9.7 ascites immunoprecipitated bands of m_r 46.8 kDa, and Pst ascites 5.2, 9.7 and 11.5 immunoprecipitated bands of m_r 112 kDa. The predicted m_r of the human enzyme is around 46 kDa(Grundmann, U., Nerlich, C., Rein, T. and Zettlmeissl, G., 1990; Stamenkovic, I., Asheim, H.C., Deggerdal, A., Blomhoff, H.K., Smeland, E.B. and Funderud, S., 1990), and in rats isoforms have been detected with m_rs of 43kDa and 47kDa(Weinstein, J., Lee, E.U., McEntee, K., Lai, P.H. and Paulson, J.C., 1987). In addition, it has been shown that in rats the enzyme probably functions as a dimer in the Golgi membrane(Fleischer, B., McIntyre, J.O. and Kempner, E.S., 1993). Taking all the evidence into account, it is possible that the Pst mAbs detect two different isoforms of β-galactoside-α-2,6-sialyltransferase with m_rs of around 47 kDa and 41 kDa. The large proteins detected at 112 kDa by immunoprecipitation may represent dimers or trimers of the enzyme (although this is speculative). The perinuclear staining pattern observed in Raji cells and in liver and pancreas sections adds further credence to the argument. α-2,6-sialyltransferase enzyme assays were performed on the Pst immunoprecipitates, but no activity was detected. However, this may have been due to the fact that protease inhibitors leupeptin, pepstatin, EDTA, TLCK-HCL and TPCK were used in the preparation of the soluble membrane samples. It has since come to light that these are potent inhibitors of sialyltransferase activity(Melkerson-Watson, L.J. and Sweeley, C.C., 1991).

If time had not been limited, I would have liked to have further defined the intracellular staining patterns of the Pst mAbs using immunoelectron microscopy techniques. I would also have liked to more accurately compare this staining pattern with that of the CDw75 mAbs by means of double-labelling techniques using electron microscopy with different sized gold particles or immunofluorescence microscopy using different coloured dyes. If the Pst mAbs can be proven to be specific for β-galactoside-α-2,6-sialyltransferase, they may have many applications in the future. Since sialylation levels, and even α-2,6-linked sialic acid levels have been shown to be directly related to tumour aggression in many cancers(Yogeeswaran, G. and Salk, P.L., 1981; Hakomori, S., Patterson, C.M., Nudelman, E. and Sekiguchi, K., 1983; Smets, L.A. and Van Beek, W.P., 1984; Dennis, J.W., 1986; Humphries, M.J., Matsumoto, K., White, S.L. and Olden, K., 1986; Cohen, A.M., Allalouf, D., Djaldetti, M., Weigl, K., Lehrer, N. and Levinsky, H., 1989; Dall'Olio, F.,

Malagolini, N., di Stefano, G., Minni, F., Marrano, D. and Serafini-Cessi, F., 1989; Francina, A., Gateau-Roesch, O., Couprie, N., Leculier, C., Col, J.F., Archimbaud, E., Campos, L., Louisot, P. and Richard, M., 1989; Springer, G.F., 1989; Bresalier, R.S., Rockwell, R.W., Dahiya, R., Duh, Q.Y. and Kim, Y.S., 1990; Easton, E.W., Bolscher, J.G.M. and van den Eijnden, D.H., 1991; Sasaki, H., Momoi, T., Yamanaka, C., Yorifuji, T., Kaji, M. and Mikawa, H., 1991; Harvey, B.E., Toth, C.A., Wagner, H.E., Steele, G.D.J. and Thomas, P., 1992; Vandamme, V., Cazlaris, H., Le Marer, N., Laudet, V., Lagrou, C., Verbert, A. and Delannoy, P., 1992), a quick and economic means of labelling and possibly quantifying α-2,6-sialyltransferase levels in tumour biopsies could prove to be of great prognostic value in the treatment of cancer patients.

CHAPTER 7

Fifth International Leucocyte Typing Workshop.

7.1 B-Cell Panel Analysis.

The function of the International Leucocyte Typing Workshop is to classify new mAb into clusters with identical binding patterns and tissue distribution and in this way assist in the identification of new markers of cell differentiation. New mAbs are sent to the workshop which distributes them in the form of panels of coded mAbs to be analysed against different cell lineages by numerous labs throughout the world. The information is collated by the workshop and the new mAbs are allocated to either existing clusters or to new unclassified clusters. I applied to the Workshop for the CDw75 and CD76 panels, but also accepted the new B-cell panel for analysis in an attempt to identify putative CDw75 mAbs. 71 mAb were sent with information limited purely to the isotype of mAb, its form (ascites or hybridoma culture supernatant) and suggested working concentration. The panel was tested immunohistochemically on normal tonsil sections and by flow cytometry on the Namalwa cell lines, some leukaemias and samples of normal peripheral blood lymphocytes. Selected mAb were also used in biochemical analyses of cell lysates in order to determine the molecular weight of the antigens to which they had been raised.

7.1.1 B-Cell Panel Analysis by Flow Cytometry.

Six Namalwa sublines, NK, CSN/70, IPN/45, PNT and KN2 (in ascending order of differentiation) were used to screen the B-cell panel. Samples of PBL from 2 normal donors and a patient with B-cell Chronic Lymphocytic Leukaemia were also used. Cells were labelled with FITC-labelled anti-mouse-Ig and analysed by flow cytometry. The results are expressed as % positive cells using gates set using isotype-matched negative controls, and are given in table 7.1.1 below. 7 mAb were missing from the panel and these have therefore been omitted from the table. mAb B000 was provided by the Workshop as a negative control, 2A4.5 and VIM13 are our own IgG and IgM matched negatives. CD19.3 was used to label the B-cell population in each sample, and DR001, an IgG2a mAb specific for MHC class II was used as a positive control. Paraffin sections of normal tonsil were also stained immunohistochemically with each mAb, and a summary of these results is included in Table 7.1.1. The results were analysed for novel patterns of reaction and for recognisable patterns of existing clusters. Approximately 40 mAb in the B-cell panel reacted preferentially with one or more of the Namalwa sublines. There were six or seven discernible patterns of expression of surface antigens, one of which was similar to that of existing cluster CD45RA, and one which was similar to that of CDw75.

Table 7.1.1 B-Cell Panel Reactivity (% Positive Cells)

mAb	NK	CSN/70	IPN/45	PNT	KN2	PBL 1	PBL 2	BCLL	Tonsil*
B000	1	1	1	1	1	3	4	2	-
2A4.5	0	0	0	1	1	2	3	1	N.D.†
VIM13	2	4	1	2	2	3	4	2	-
CD19.3	99	87	78	99	82	14	9	98	N.D.
DR001	45	59	100	99	100	18	20	99	N.D.
B01	1	5	27	36	77	12	9	44	-
B02	3	2	2	3	3	0	3	2	+
B03	1	2	1	3	3	4	4	1	+ dendritic
B04	1	2	1	1	1	4	3	1	+ dendritic
B05	1	2	1	1	1	3	3	1	+
B06	34	85	100	99	99	63	74	86	+/-
B07	2	1	1	2	1	14	4	86	+/-
B08	53	94	93	99	59	7	6	86	++
B09	0	1	0	0	1	2	4	1	-
B10	47	34	99	90	72	21	17	99	+
B11	8	22	2	5	13	1	4	3	+
B12	1	3	67	40	5	33	60	97	-
B14	0	2	0	1	3	2	4	2	-
B15	0	1	1	1	2	1	2	1	-
B16	100	98	99	100	100	1	2	1	+
B17	0	2	1	1	2	1	3	1	-
B18	0	1	0	1	1	1	3	1	-
B19	100	100	100	100	100	96	94	38	+/-
B20	1	2	3	5	1	11	9	0	-
B21	1	1	1	1	1	1	4	1	-
B24	1	2	0	1	0	1	4	1	-
B25	93	89	100	98	89	9	70	98	+ follicular
B27	3	5	60	26	6	29	57	98	-
B28	4	3	1	2	6	9	8	1	-
B29	6	50	84	38	51	8	10	2	++ follicular
B31	34	75	94	99	97	1	26	36	-
B32	32	69	92	99	96	17	25	58	+/-

mAb	NK	CSN/70	IPN/45	PNT	KN2	PBL 1	PBL 2	BCLL	Tonsil*
B34	0	1	0	1	3	1	3	1	-
B35	1	1	0	1	9	1	2	1	-
B36	14	49	6	15	20	12	8	92	+/-
B37	2	5	47	74	17	7	9	4	-
B38	4	9	0	2	2	42	60	1	-
B39	0	0	0	1	1	1	3	1	+/-
B40	6	10	20	6	67	1	3	3	++
B41	99	96	99	99	94	4	21	96	-
B42	0	0	0	1	0	1	3	1	-
B43	3	0	0	1	12	0	3	1	-
B44	32	81	100	100	81	36	76	98	+
B46	4	55	13	28	7	1	4	1	+
B47	0	0	0	1	1	10	9	10	-
B48	99	99	95	86	11	97	97	99	++
B49	1	2	14	34	2	9	7	1	-
B50	0	1	0	1	1	12	7	1	+/-
B51	0	1	0	1	0	3	4	1	-
B52	89	87	28	6	48	9	7	1	-
B53	1	1	0	1	0	3	4	2	-
B54	64	79	94	99	99	54	32	95	-
B55	93	87	67	94	16	57	54	83	++
B56	100	99	98	100	91	96	97	100	+ T-cell areas
B57	70	57	40	89	29	32	28	22	+ T-cell areas
B59	100	100	99	95	99	97	98	100	+
B60	0	0	0	1	0	3	3	1	-
B62	31	94	100	100	99	75	78	98	+ dendritic
B63	29	93	100	100	99	75	78	97	+ T-cell areas
B64	37	95	93	96	59	4	4	87	+
B65	20	20	1	2	1	4	4	2	+/-
B66	16	23	1	1	2	4	3	1	-
B67	100	100	100	100	100	97	96	31	-
B68	100	100	100	100	100	97	95	36	+/-
B69	100	100	100	100	100	98	97	54	+/-

mAb	NK	CSN/70	IPN/45	PNT	KN2	PBL 1	PBL 2	BCLL	Tonsil*
B70	100	100	100	100	100	97	96	67	-
B71	100	100	100	100	100	96	95	10	+

* - indicates no staining; + indicates general staining unless further information given.
†N.D.= not determined

mAb pairs with similar patterns and those mAbs of the panel which produced staining patterns similar to those of existing clusters are listed in table 7.1.2. Pairs producing similar staining patterns were B006 and B044, B008 and B064, B012 and B027, B019 and B071, B031 and B032, B065 and B066, B067 and B068, and B069 and B070. The mAbs B019, B071, B067, B068, B069 and B070 were all similar in reactivity.

Table 7.1.2 Potential Cluster Members in B-cell panel.

	Percentage positive cells								*
mAb	NK	CSN70	IPN45	PNT	KN2	PBL1	PBL2	B-CLL	Reactivity
B006	34	85	99	99	99	63	74	86	CD45RA
B044	32	81	99	99	81	36	76	98	CD45RA
B008	53	94	92	98	59	7	6	86	UC
B064	37	95	93	96	59	4	4	87	UC
B012	1	3	67	40	5	33	60	97	?CDw75
B027	3	5	60	26	6	29	57	98	?CDw75
B019	99	99	99	99	99	96	94	38	PC#2
B071	99	99	99	99	99	96	95	10	?PC#2
B031	34	75	94	99	97	1	26	36	UC
B032	32	69	92	99	96	17	25	58	UC
B065	20	20	1	2	1	4	4	2	PC#1
B066	16	23	1	1	2	4	3	1	PC#1
B067	99	99	99	99	99	97	99	31	PC#2
B068	99	99	99	99	99	97	95	35	PC#2
B069	99	99	99	99	99	98	97	54	PC#2
B070	99	99	99	99	99	97	96	67	PC#2

* UC, unclustered mAb; PC, possible cluster.

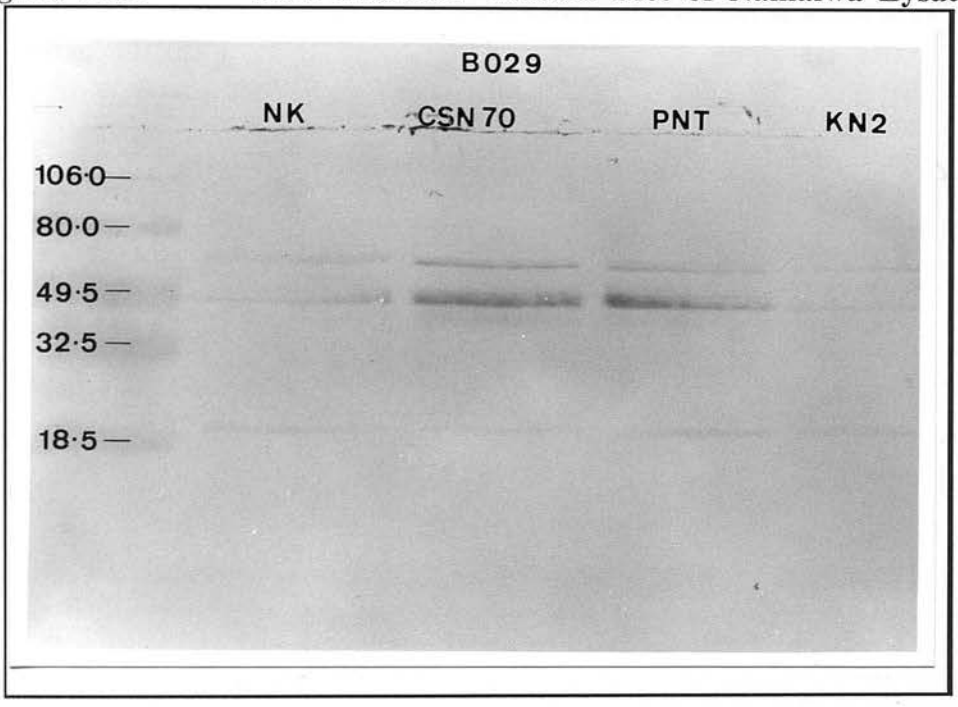
Antibodies B012 and B027 have a similar expression pattern to CDw75, although the level of binding on PNT cells is a bit low. This work is published in the Workshop

Proceedings in which the codes for all mAbs are listed. mAb B012 is the possible CDw75 mAb EBU-65(Ross, J.A. and Andrew, J.M., 1995).

7.1.2 Biochemical Analysis of B-cell Panel.

Selected mAb which had successfully labelled cells immunohistochemically and flow cytometrically were used in basic biochemical studies. All mAbs producing any level of staining were used to immunoprecipitate Ag from PNT cell lysates.

Figure 7.1.1 B029 Labelled Western Blot of Namalwa Lysates.



Immunoprecipitates were separated electrophoretically and transferred to nitro-cellulose by Western blotting. Protein bands were detected using India Ink. Weak bands were detected in precipitates of B29, B71 and B55. Unfortunately, the bands were not clear enough to calculate any molecular weights. The results were therefore used merely as an indicator of potential binding activity to denatured protein. Lysates were then made of 5 of the Namalwa sublines and these were separated on 3 individual SDS polyacrylamide gels, transferred to nitro-cellulose by Western blotting and each probed with one of the above 3 mAb and developed using the DAB/HRP method. Only B29 produced positive protein bands as shown in figure 7.1.1. Double bands of molecular weights ~50 and 60kD were detected. These were of highest intensity in cell lines CSN/70 and PNT, and more weakly in NK and KN2. This reflects to a certain extent, the extracellular binding detected on these cell lines by flow

cytometry, although the intensity of bands detected in KN2 cell lysates is not as high as would be predicted from the flow cytometry results. This may be due to a decrease in intracellular antigen in these more differentiated cells as compared to high intracellular and extracellular antigen levels in CSN/70 and PNT cells. IPN/45 lysates were not used in this analysis.

7.2 CDw75 & CD76 Panel Analysis.

CDw75 and CD76 mAb panels were specifically requested from the Workshop. At the Fourth International Leucocyte Typing Workshop, CDw75 mAbs were shown to be mainly reactive with mature sIg^+ cells of the B-cell lineage, and with germinal centre B-cells in solid lymphoid tissue(Dorken, B., Moller, P., Pezzutto, A., Schwartz-Albiez, R. and Moldenhauer, G., 1989). Only a limited amount of information about the biochemical nature of the antigen was described at this workshop, with conflicting results of molecular weight determinations indicating molecular weights of 53 kDa (Dorken, B., Moller, P., Pezzutto, A., Schwartz-Albiez, R. and Moldenhauer, G., 1989) and 87 kDa (Mittler, R.S., Talle, M.A., Carpenter, K., Rao, P.E. and Goldstein, G., 1983) for the OKB4 antigen. In addition, the LN-1 epitope has been shown to be destroyed by neuraminidase treatment(Epstein, A.L., Marder, R.J., Winter, J.N. and Fox, R.I., 1984). However, no further information regarding epitope structures was generated at the previous workshop. Since the last workshop, CDw75 and CD76 have both been shown to be generated by the activity of β-galactoside-α-2,6-sialyltransferase(Bast, B.J., Zhou, L.J., Freeman, G.J., Colley, K.J., Ernst, T.J., Munro, J.M. and Tedder, T.F., 1992). However, previous experiments with the CDw75 mAbs (chapter 3) have shown that not all epitopes are sialylated, and I wanted the opportunity to repeat these experiments using fresh mAbs and to compare CDw75 epitope structures with those of CD76. Tunicamycin was used to inhibit N-linked glycosylation of $CDw75^+$ and $CD76^+$ cells, and neuraminidase was used to eliminate sialic acid. Expression of Ag of both clusters on Namalwa sublines was also compared.

7.2.1 Titration of CDw75 and CD76 mAb on PNT cells Before and After Neuraminidase Treatment.

Initial titrations were carried out on both sets of mAb and on previously obtained CD76 mAb HD66 to establish optimum concentrations for cell-labelling. Dilutions of

1/100, 1/200, 1/500 and 1/1000 were used to label PNT cells. Half of the samples of PNT cells were untreated and the other half were pre-treated with neuraminidase to remove sialic acid. FITC-labelled secondary mAb was used and fluorescence detected by flow cytometry. Results are described as % positive cells (over a threshold determined using isotype-matched negative controls). The results are given in Table 7.2.1 below.

Table 7.2.1 CDw75 and CD76 Titration on PNT Cells. (% Positive Cells)

	1/100		1/200		1/500		1/1000	
mAb	-N*	+N	-N	+N	-N	+N	-N	+N
CDw75.1	66	99	61	98	50	**97**	44	94
CDw75.2	99	7	100	7	100	4	**99**	8
CDw75.3	100	5	99	7	99	5	**96**	5
CDw75.4	90	16	94	12	91	11	**91**	8
CDw75.5	95	6	**92**	7	85	4	76	6
CD76.1	95	5	**92**	5	86	4	73	5
CD76.2	**94**	90	72	55	36	22	13	9
CD76.3	86	100	80	100	64	100	49	**99**
CD76.4	74	48	84	54	90	65	**90**	70
HD66	**38**	5	22	4	7	7	4	7
VPM30	1	3	2	3	1	4	1	4

* -N indicates no treatment of cells before staining,
+N indicates pre-treatment with neuraminidase.

The minimum concentrations of each mAb required for optimal binding are represented by shading. These are the concentrations which were used in all subsequent experiments. Table 7.2.2 gives the comparative binding levels of the four CDw75 mAb used in all other chapters at their current activity levels. As previously mentioned, the activity of EBU-65 stocks would appears to have deteriorated with storage when compared with the activities of the fresh workshop mAb.

Table 7.2.2 CDw75 Expression on PNT cells.(% Positive Cells)

mAb	HH2	OKB4	EBU-141	EBU-65
-N	94	29	93	38
+N	7	75	8	5

Neuraminidase sensitivity is a good indicator of the glycosylation state of each epitope (as previously discussed). Only one CDw75 epitope (CDw75.1) appeared to be masked by sialic acid. The number of positive cells detected by this mAb after neuraminidase treatment was increased by 94% at the optimum mAb concentration of 1/500. mAb CDw75.1 was therefore identified as OKB4. All other CDw75 epitopes were destroyed by neuraminidase treatment, thus confirming previous findings.

With the CD76 mAbs, the CD76.3 epitope was found to exhibit the same phenomenon as CDw75.1, in that it is also masked by sialic acid. The number of cells recognised by this mAb was also increased by 103% after treatment with neuraminidase. CD76.1 and HD66 binding was completely abrogated by neuraminidase treatment, indicating that this epitope is sialylated. However, neuraminidase treatment had little or no effect on CD76.2 and CD76.4 binding.

7.2.2 Investigation of N-Linked Glycosylation of Epitopes.

Neuraminidase treatment provided an assessment of sialic acid involvement in each epitope. However, if CDw75 expression is under the control of the enzyme β-galactoside-α-2,6-sialyltransferase as has been reported, then it would seem logical to determine if each epitope contains the N-linked glycosides necessary for this enzyme to act. As previously explained in chapter 3.2, tunicamycin is a potent inhibitor of N-linked glycosylation. If tunicamycin treatment causes a reduction in epitope recognition by any of the mAb studied, then we can assume that the epitopes recognised are expressed on N-linked carbohydrate.

As in chapter 3.2, PNT cells were used in this study. Cells were harvested from culture, stripped of their sialic acid by treatment with neuraminidase, and then cultured overnight in varying concentrations of tunicamycin. Monensin was not used to inhibit re-sialylation in this study. Instead, where de-sialylation was deemed necessary (i.e. for sialic-acid-masked epitopes CDw75.1 and CD76.3), neuraminidase treatment was repeated before staining with these mAb. Cells were labelled with all mAb from both clusters, and also with the previously obtained samples of identified CDw75 mAb and the lectins SNA and MAA (which provide a gauge of glycosylation state). The experiment was repeated four times and gave consistent levels of staining for each

mAb in each repetition. The results of these four experiments were pooled, and mean percentages of positive cells are given in table 7.2.3 below.

Table 7.2.3 Results of Tunicamycin Inhibition of PNT-Cell Glycosylation on CDw75 & CD76 Expression (% Positive Cells).

mAb	Before treatment		After treatment				
	-N	+N	0tm*	0.25tm	0.5tm	1.0tm	2.0tm
CDw75.1	39	87	55	57	58	54	54
CDw75.1(N)†		87	81	84	74	70	66
CDw75.2	84	1	64	56	34	25	17
CDw75.3	69	1	48	41	24	16	10
CDw75.4	43	16	36	31	22	22	19
CDw75.5	42	1	26	17	8	4	2
CD76.1	32	1	19	11	7	5	4
CD76.2	29	37	54	33	38	32	26
CD76.3	2	95	66	77	77	78	77
CD76.3(N)		95	90	89	87	90	95
CD76.4	74	61	73	69	62	60	57
HH2	47	0	34	24	14	8	6
OKB4	19	49	28	26	24	23	22
OKB4(N)		49	42	45	38	34	28
EBU-141	45	16	36	31	24	25	19
EBU-65	7	1	6	3	2	2	2
SNA	99	36	90	82	53	36	25
SNA(N)		36	34	33	28	24	10
MAA	60	97	91	100	92	90	77
MAA(N)		97	94	99	92	94	87

*tm = tunicamycin concentration used in µg/ml

† (N) indicates cells re-treated with neuraminidase

SNA binding is a good indicator of N-linked glycosylation levels as it specifically binds to sialic acid in the α-2,6-linkage on N-linked carbohydrate(Shibuya, N., Goldstein, I.J., Broekaert, W.F., Nsimba-Lubaki, M., Peeters, B. and Peumans, W.J., 1987). As can be seen from table 7.2.3 above, N-linked glycosylation of these cells was indeed inhibited by tunicamycin treatment. The results also show that the level of inhibition was directly proportional to the concentration of tunicamycin used.

MAA binds to O-linked glycans, and the results also show that tunicamycin treatment had little or no effect on O-linked glycosylation.

It can be seen from table 7.2.3, that the most noticeable effect of tunicamycin on CDw75 and CD76 expression was on epitopes CDw75.2, CDw75.3, CDw75.4, CDw75.5 and CD76.1. Tunicamycin was observed to have no effect on the CD76.3 epitope (after neuraminidase treatment), and there was only a moderate reduction in the numbers of cells recognised by CDw75.1, CD76.2 and CD76.4 after the treatment.

These results indicate that epitopes CDw75.2, CDw75.3, CDw75.4, CDw75.5 and CD76.1 are all composed of N-linked carbohydrate. CDw75.1, CD76.2 and CD76.4 epitopes may partially consist of N-linked carbohydrate, and the moderate binding levels observed after tunicamycin treatment may be due to reduced affinity of binding of the mAb to partial epitopes. CD76.3 binding was completely unaffected by the treatment, indicating that this epitope does not contain any N-linked carbohydrate. Another explanation for the observed differences may be that the epitopes are also expressed on glycolipids. CD76 mAbs have been shown to identify a carbohydrate antigen present on gangliosides(Bast, B.J., Zhou, L.J., Freeman, G.J., Colley, K.J., Ernst, T.J., Munro, J.M. and Tedder, T.F., 1992). Individual CD76 mAbs have also been shown to produce different staining patterns dependent on the polarity of the gangliosides stained. It is also possible that CDw75 epitopes are present on glycolipids in addition to glycoproteins. This theory is supported by the observation that tunicamycin does not produce 100% inhibition of synthesis of any of the epitopes studied.

It was noticed that the CD76.1 epitope structure is more similar to that of the CDw75.2, CDw75.3, CDw75.4 and CDw75.5 epitopes than to the other CD76 epitopes. It should also be noted that the CDw75.1 epitope seems more similar in structure to the CD76.2 and CD76.4 epitopes than to the other CDw75 epitopes. This will be discussed more fully in section 7.3.

7.2.3 CDw75 and CD76 Expression on Namalwa Sublines.

CDw75 and CD76 mAb were screened against the six available Namalwa sublines, G4, NK, CSN/70, IPN/45, PNT and KN2 (as described in Chapter 3) in order to compare the relative distribution of both antigens on B-cells at various stages of

differentiation. Cells were harvested and stained as before using FITC-labelled secondary mAb, and analysed by flow cytometry. Results are expressed as percentages of positive cells above a threshold determined by isotype matched negative controls, and are given in table 7.2.4 below. Namalwa sublines are presented in ascending order of differentiation. Where indicated, cells were treated with neuraminidase prior to staining. These results are published in the Workshop Proceedings(Andrew, J.M. and Ross, J.A., 1995).

Table 7.2.4 CDw75 & CD76 Expression on Namalwa Sublines (% positive cells)

mAb	G4	NK	CSN/70	IPN/45	PNT	KN2
HH2	7	6	1	75	94	6
OKB4 (N)*	46	9	9	63	75	8
EBU-141	8	2	1	66	93	2
EBU-65	8	1	1	19	38	1
CDw75.1	6	2	1	51	50	2
CDw75.1 (N)	55	15	32	85	97	17
CDw75.2	9	16	3	92	99	19
CDw75.3	9	9	2	78	96	13
CDw75.4	6	2	1	63	91	3
CDw75.5	7	2	2	59	92	5
CD76.1	9	4	2	17	92	14
CD76.2	43	19	14	70	94	61
CD76.3	10	1	1	7	49	4
CD76.3 (N)	76	69	54	92	99	99
CD76.4	98	36	80	18	90	9
HD66	9	2	1	4	38	5
VPM30	2	1	1	1	1	1
2A4.5	4	2	1	2	1	2

As previously established, all CDw75 epitopes were found on sublines IPN/45, and PNT. These are the more mature sublines and are sIg^+. Four of the CDw75 mAb did not label the immature B-cell precursors or the most mature KN2 cells to any great extent. The OKB4 (CDw75.1) epitope however was found on the very immature G4 cells and on the remaining three sublines (NK, CSN/70 & KN2) to a lesser extent.

This suggests that the OKB4 epitope is expressed at different stages of cell differentiation from the other epitopes. The distribution of this epitope on Namalwa sublines actually more closely resembles that of the CD76 epitopes with the exception of CD76.1 which in turn resembles the distribution of the other CDw75 epitopes. In general the CD76 epitopes CD76.2, CD76.3 and CD76.4, like the CDw75.1 epitope were expressed on all cells, but to a greater extent on the more mature cell lines IPN/45, PNT and KN2 (with the exception of CD76.4).

7.3 Discussion.

Both CDw75 and CD76 have previously been shown to be expressed mainly on mature B-cells and on a subset of T-cells. In my analysis, I have concentrated mainly on cells of B-cell origin, and have confirmed that the antigens are indeed most strongly expressed on the more mature B-cells. In the case of CDw75, very little structural data is available for each of the epitopes, although it is assumed that they all consist of α-2,6-sialylated structures on N-linked carbohydrate(Munro, S., Bast, B.J., Colley, K.J. and Tedder, T.F., 1992). However, as shown in chapter 3 and confirmed in this chapter, this cannot be the case for all epitopes of CDw75 since the OKB4 epitope is in fact masked by sialic acid, as is that of CD76.3. It is difficult to understand how transfection of cDNA for β-galactoside-α-2,6-sialyltransferase into COS cells can give rise to these non-sialylated epitopes, unless expression of this enzyme in some way controls the expression of another glycosyltransferase enzyme which produces the epitopes recognised by these mAbs.

To add a further complication, it is also possible that these epitopes are not only expressed on glycoproteins, but are also expressed on glycolipids. As discussed in chapter 5, this may explain why it has been so difficult to isolate CDw75 by biochemical means. There is a growing body of evidence which suggests that glycosyltransferase enzymes and in particular the α-2,6-sialyltransferase enzyme are also capable of glycosylating gangliosides of the glycolipids in cell membranes(Hakomori, S., Patterson, C.M., Nudelman, E. and Sekiguchi, K., 1983; Feizi, T., 1985; Dall'Olio, F., Malagolini, N., di Stefano, G., Minni, F., Marrano, D. and Serafini-Cessi, F., 1989; Paulson, J.C. and Colley, K.J., 1989; Kanani, A., Sutherland, D.R., Fibach, E., Matta, K.L., Hindenburg, A., Brockhausen, I., Kuhns, W., Taub, R.N., van den Eijnden, D.H. and Baker, M.A., 1990; Gottfries, J., Percy, A.K., Mansson, J.E., Fredman, P., Wikstrand, C.J., Friedman, H.S.,

Bigner, D.D. and L, S., 1991; Sasaki, H., Momoi, T., Yamanaka, C., Yorifuji, T., Kaji, M. and Mikawa, H., 1991; Erikstein, B.K., Funderud, S., Beiske, K., Aas-Eng, A., De Lange Davies, C., Blomhoff, H.K. and Smeland, E.B., 1992; Shah, S., Lance, P., Smith, T.J., Berenson, C.S., Cohen, S.A., Horvath, P.J., Lau, J.T. and Baumann, H., 1992). Indeed, CD76 mAbs have already been shown to bind to both glycolipids and glycoproteins(Bast, B.J., Zhou, L.J., Freeman, G.J., Colley, K.J., Ernst, T.J., Munro, J.M. and Tedder, T.F., 1992), and binding has also been shown to be dependent on the polarity of the gangliosides. The fact that tunicamycin does not produce 100% inhibition of synthesis of any of the CDw75 or CD76 epitopes suggests that they may be expressed on both N-linked carbohydrate and on glycolipids. This may also explain the variation in binding levels observed with the different mAbs.

Another controversial result arising from this study is the fact that the OKB4 binding pattern on Namalwa cells seems to more closely resemble that of the CD76 mAbs CD76.2 - CD76.4 than that of the other CDw75 mAbs. Unlike the other CDw75 mAbs, OKB4 stains the very immature B-cell subline G4, and to a lesser extent, sublines NK, CSN/70 and KN2. This expression is only detected after neuraminidase treatment, but the antigen is expressed on these cells never-the-less. This is more like the pattern of expression observed with the CD76 mAbs. Ironically, it was also observed that CD76.1 produces a staining pattern which is more akin to that of the remaining CDw75 mAbs than to the other CD76 mAbs, as it only stains mature B-cell sublines IPN/45 and PNT. CD76.1 binding is also completely abolished by neuraminidase treatment unlike the other CD76 mAbs. Since the two offending mAbs are coded CDw75.1 and CD76.1 it is possible that there was some mix up in their labelling at a previous workshop, and that they have been allocated to the wrong clusters. Admittedly, further proof in the form of detailed immunohistochemical characterisation of each antigen on a variety of tissues will have to be sought in order for this matter to be cleared up satisfactorily. There is also a case against epitopes CDw75.1 and CD76.3 being included in either cluster due to the fact that they cannot possibly be generated by the activity of the enzyme α-2,6-sialyltransferase, since sialylation of these epitopes actually inhibits mAb binding.

CDw75 and CD76 are not the only carbohydrate containing clusters. To give but two examples, leukosialin (CD43) is a major cell-surface sialo-glycoprotein of thymocytes and T-cells(Cyster, J.G., Shotton, D.M. and Williams, A.F., 1991), and CD44 is another glycoprotein expressed on leucocytes and red blood cells(Spring, F.A.,

Dalchau, R., Daniels, G.L., Mallinson, G., Judson, P.A. and Parsons, S.F., 1988). As carbohydrate plays such a major role in antigen recognition, the question of "what defines a cluster of differentiation?" must surely be addressed. Since the protein content of such carbohydrate-containing clusters often remains unknown or unidentifiable, and in the case of CDw75 and CD76, where some mAbs of the cluster do not recognise antigen until sialic acid has been removed, how can we be sure that the antigen recognised is the same for all mAbs of the cluster? In addition, if a mAb is found to be specific for a carbohydrate moiety which is expressed on a number of different glycoproteins and glycolipids whose individual expression is also developmentally regulated, how can this carbohydrate antigen be allocated to a cluster? As it becomes more accepted that the role of carbohydrate is of major importance in a number of functions such as receptor-binding, antigen recognition, growth regulation and development, fundamental questions must be asked as to the entire basis of the International Workshop, and to the criteria which define a cluster of differentiation.

CHAPTER 8

Conclusions.

8.1 Tissue distribution of CDw75.

It had previously been established that all CDw75 epitopes were expressed predominantly on the cell-surface of mature sIg^+ B-cells and on B-cell leukaemias and lymphomas(Dorken, B., Moller, P., Pezzutto, A., Schwartz-Albiez, R. and Moldenhauer, G., 1989), and these findings have been confirmed in this project. However, very little was known about CDw75 expression outwith the mature B-cell population, and about the structure of the CDw75 epitopes. I have shown that the CDw75 mAbs do not recognise a single epitope, and that different epitopes are expressed at different levels on human peripheral blood T-cells. Epitopes recognised by HH2 and OKB4 are induced when T-cells are activated, whereas epitopes recognised by EBU-141 and EBU-65 are expressed on resting T-cells and expression of these epitopes is reduced upon T-cell activation.

It was also known that CDw75 was expressed in the B-cell areas of lymphoid tissues, and particularly in follicular areas. In this study it has also been shown that CDw75 is expressed in other tissues such as liver, pancreas, breast and intestine. In normal liver, all mAbs preferentially stained the canaliculae. It is known that there are receptors for terminally galactosylated sugars in the liver, and it is therefore possible that the epitopes recognised by CDw75 have been sialylated in the liver and secreted into the canaliculae.

In samples of intestinal tissue, CDw75 was only detected on malignant cells. Others have observed increased levels of α-2,6-sialyltransferase activity in colon cancer cells(Dall'Olio, F., Malagolini, N., di Stefano, G., Minni, F., Marrano, D. and Serafini-Cessi, F., 1989; Bresalier, R.S., Rockwell, R.W., Dahiya, R., Duh, Q.Y. and Kim, Y.S., 1990), which may explain this increase in CDw75 expression. CDw75 expression was also detected in breast cancer samples. As previously discussed, it was expected that CDw75 expression would be increased in tumour samples as there are many accounts of increased sialylation of glycoproteins and glycolipids in cancer tissues. The results obtained using the Pst mAbs on liver and pancreas sections did not show increased staining levels on tumour tissues in comparison with normal tissues, however the samples used were from a variety of patients with different types of tumours, and so these results should be regarded as preliminary. It would have been of great interest to obtain a supply of breast and colon cancer specimens of the same type, and to compare sialyltransferase expression on these with normal tissue samples.

8.2 Epitope structure.

It was previously thought that all CDw75 mAb recognised a single epitope(Dorken, B., Moller, P., Pezzutto, A., Schwartz-Albiez, R. and Moldenhauer, G., 1989). In this study, it has been conclusively proved that this is not the case. Epitopes HH2, EBU-141 and EBU-65 all contain sialic acid, whereas the OKB4 epitope is masked by sialic acid, although it does contain N-linked carbohydrate. In addition, as mentioned above, epitopes are expressed on different subsets of T-cells. It was therefore concluded that the four mAbs studied here do not recognise a single epitope.

Another question raised by this study is whether CDw75 is expressed on glycoproteins or on glycolipids. Clearly, the epitope is expressed on at least one glycoprotein as its expression is inhibited by tunicamycin which inhibits N-linked glycosylation. However, the same glycosytransferase enzyme can glycosylate both glycoproteins and glycolipids, and it is possible that it is expressed on many different glycoproteins and glycolipids, as no specific protein band was detected with the CDw75 mAbs. The fact that the antigen is stable on paraffin sections also indicates that it may be expressed on glycolipid.

8.3 Pst mAbs.

The Pst mAbs produced in this study are more than likely to be specific for β-galactoside-α-2,6-sialyltransferase as they are specific for the JP3 peptide. In addition, they exhibit perinuclear staining of RAJI cells, liver cells and pancreas cells in the Golgi area of the cell which is where the sialyltransferase is mainly located. Furthermore, in immunoprecipitation experiments, the mAbs precipitate a protein of molecular weight 47 kDa, and in Western blotting, pick out bands of 41 kDa and 47 kDa. The predicted molecular weight for the human enzyme is around 46 kDa(Grundmann, U., Nerlich, C., Rein, T. and Zettlmeissl, G., 1990), and it is expected that there are different isoforms of the enzyme, as in rats different forms have been detected with molecular weights of 43 kDa and 47 kDa(Weinstein, J., Lee, E.U., McEntee, K., Lai, P.H. and Paulson, J.C., 1987).

8.4 Leucocyte Typing Workshop.

At the Fifth International Leucocyte Typing Workshop, it was acknowledged that the OKB4 epitope is not the same as the other CDw75 epitopes, and that all epitopes have slightly different distributions(Engel, P. and Tedder, T.F., 1995). It was also noted that the OKB4 epitope may have to be removed from the cluster, but not until the cluster is biochemically defined. It was also acknowledged that there is considerable overlap between the CDw75 and CDw76 epitopes, all of which I have found in this study.

From this study, it is clear that the basis for defining a CD antigen needs to be clarified. If the same carbohydrate group can be expressed on many different glycoproteins and glycolipids which are expressed at different stages of cell development, how can this be defined as a single antigen? In addition, if a cluster is defined by the maturity of the cells on which it is expressed, how can a carbohydrate determinant be included in this classification if it is expressed on different cell-surface components at different stages of differentiation? It may be that the glycosyltransferase enzymes are developmentally regulated, and that specific carbohydrates are only expressed at certain times, but it would seem logical that there should be a separate workshop for these carbohydrate antigens which would address all the issues discussed here.

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APPENDIX A (RECIPES).

A1 Phosphate Buffered Saline (PBS) pH7.3.

8g NaCl

0.2g KCl

1.15g Na_2HPO_4 (anhydrous)

0.2g KH_2HPO_4 (anhydrous)

1 litre distilled H_2O

Concentrated HCl to adjust pH to 7.3.

A2 Lysis Buffer.

10mM Tris HCl pH 7.4

A3 Table A1 - Inhibitor concentrations.

Protease inhibitor	Working concentration	(molarity)	Glycosidase inhibitor	Working concentration	(molarity)
PMSF*	174 µg/ml	(1 mM)	M-α-D-P*	19.42 mg/ml	(0.1 M)
EDTA*	37 µg/ml	(100 µM)	DMNJ*	80 µg/ml	(400 µM)
Leupeptin	0.5 µg/ml	(1 µM)	DNJ*	65.6 µg/ml	(400 µM)
Pepstatin A	0.7 µg/ml	(1 µM)	swainsonine	1.7 µg/ml	(10 µM)
TLCK-HCl*	50 µg/ml	(135 µM)	yeast mannan	15 mg/ml	-
TPCK*	100 µg/ml	(284 µM)			

*PMSF = Phenylmethanesulphonyl fluoride, EDTA = Ethylenediaminetetraacetic acid, TLCK-HCl = L-1-Chloro-3-[4-tosylamido]-7-amino-2-heptanone-HCl, TPCK = L-1-Chloro-3-[4-tosylamido]-4-phenyl-2-butanone, M-α-D-P = methyl-α-D-pyranoside, DMNJ = Deoxymannojirimycin, DNJ = Deoxynojirimycin.

A4 Immunoprecipitation buffers.

A4.1 High Salt Buffer.

25mM Tris (pH7.4)

0.65M NaCl

1% detergent

0.05% SDS

A4.2 Low Salt Buffer.

25mM Tris (pH7.4)

0.15M NaCl

1% detergent

0.05% SDS

A5 Reducing Sample Buffer for SDS PAGE Samples.

1ml 0.5M Tris-HCl pH 6.8

0.8 ml glycerol

1.6 ml 10% SDS in distilled water

0.4 ml β-mercapto ethanol

0.2 ml 0.05% Bromophenol blue

4ml distilled water

A6 Tris Buffered Saline (TBS)

100ml 0.125 M TRIS pH7.6

900ml 8.5g/l NaCl

A7 ABC Buffer (Tris-HCl pH 7.6).

24ml 0.2M Tris

38ml 0.1N HCl

38ml distilled water.

A8 Immunohistochemistry / Cell-Bound ELISA Substrate Solutions.

A8.1 Peroxidase Substrate.

!mg/ml DAB (Sigma) in TBS

20µl/ml 1 % H_2O_2.

A8.2 Alkaline Phosphatase Substrate.

Vector Red alkaline phosphatase substrate kit (Vector Laboratories).

A9 α-2,6-Sialyltransferase Assay / HRP ELISA Substrate.

A9.1 Substrate solution:

5mg OPD (Sigma)

5µl 30% H_2O_2

12.5 ml citrate buffer.

9.2 Citrate buffer:

25.7ml 0.2M Na_2PO_4

24.3ml 0.1M citric acid

50ml distilled H_2O.

A10 SDS PAGE Tank Buffer.

3g Tris base

14.4g Glycine

1g SDS

Make to 1l with distilled water

A11 SDS PAGE Transfer Buffer.

5.8g Tris base

2.9g Glycine

0.37g SDS

200ml Methanol

Make up to 1l with distilled water.

A12 Western Blot DAB Substrate Solution.

1mg/ml DAB(Sigma - tablets)

1µl/ml 30% H_2O_2

4µl/ml 80 mg/ml Cobalt Chloride.

A13 Alkaline Phosphatase ELISA Substrate.

1mg/ml paranitrophenyl phosphate (Sigma)

0.1M glycine-HCl pH 10.4

0.001M $MgCl_2$

0.001M $ZnCl_2$

APPENDIX B.
PUBLICATIONS ARISING FROM THIS WORK.

Andrew, J. M. and Ross, J. A. (1995). Modulation of CDw75 and CD76 following neuraminidase and tunicamycin treatments. Leucocyte Typing V., Boston, USA, Oxford University Press.

Guy, K. and Andrew, J. M. (1991). "Expression of the CDw75 (beta-galactoside alpha 2,6-sialyltransferase)antigen on normal blood cells and in B-cell chronic lymphocytic leukaemia." Immunology. **74**(2): 206-214.

Ross, J. A. and Andrew, J. M. (1995). Potential cluster analysis with variant sublines of Namalwa. Leucocyte Typing V., Boston, USA, Oxford University Press.

B16.4 Modulation of CDw75 and CD76 following neuraminidase and tunicamycin treatments

J. ANDREW and J. A. ROSS

CDw75 is a cell-surface antigen that was assigned at the Fourth Workshop where four mAb, HH2, OKB4, LN1, and EBU-141, were clustered. A fifth mAb, EBU-65 was also found to exhibit CDw75-like staining. The gene cloned by Stamenkovic using the COS cell system and the CDw75 mAb HH2 was found to be almost identical in sequence to that of the human β-galactoside-α-2,6-sialyltransferase. This enzyme catalyses the transfer of sialic acid from its sugar-nucleotide donor CMP-NeuAc to terminal GlcNAc-β-1,4-galactose groups on N-linked carbohydrate. CDw75 and CD76 epitopes were later suggested to be the glycosylation products of this enzyme [1] acting on COS cell native proteins. This study identifies the effect of neuraminidase and tunicamycin on the expression of these structures.

The CDw75 and CD76 antibodies exhibited non-coordinate expression on the variant sublines of Namalwa. The Namalwa sublines are, in increasing order of phenotypic maturity, G4→NK→CSN/70→IPN/45→PNT→KN2. G4 and NK display some lineage infidelity (for example, CD7 expression), CSN/70 has no surface Ig, PNT has surface Ig, and KN2 secretes Ig. CDw75.2, CDw75.3, CDw75.4, and CDw75.5 reactivity was absent from G4 and NK, increased slightly on CSN/70, was high on IPN/45 and PNT, and was low on KN2. Expression of the CDw75.1 structure was moderate on IPN/45 and PNT but was absent from all the other Namalwa lines. However, neuraminidase treatment (*Clostridium perfringens*) revealed CDw75.1 expression on G4 (moderate), NK and CSN/70 (low→moderate), IPN/45 and PNT (high), and KN2 (low).

Staining with CDw75.2, CDw75.3, CDw75.4 and CDw75.5 was markedly reduced or abolished following neuraminidase treatment of the Namalwa variant line PNT (Table 1). The expression partially recovered overnight in culture, which may indicate rapid turnover of the host molecule. The expression paralleled that seen after staining with *Sambucus nigra* lectin which

Table 1 CDw75 and CD76 expression on the Namalwa variant subline PNT following neuraminidase and tunicamycin treatments

Workshop mAb		Percentage positive cells*						
		Day 0		Tunicamycin concentration (μg/ml)				
Code	Clone name	Control†	NANA‡	Nil§	0.25¶	0.5¶	1.0¶	2.0¶
CDw75.1	OKB4	45	92	70	65	65	54	56
CDw75.2	HH2	90	2	70	54	31	16	6
CDw75.3	LN1	77	1	54	39	19	7	2
CDw75.4	EBU-141	48	20	41	28	17	13	8
CDw75.5	1ZB55	48	3	30	16	5	1	1
CD76.1	CRIS-4	39	1	21	9	3	2	1
CD76.2	HD66	23	56	19	7	4	2	1
B058	BL-OFX/B8	4	99	81	90	88	90	86
B057	8H7	87	73	71	65	55	53	50
SNA‖		99	8	81	66	35	14	2

*Indirect immunofluorescence using rabbit anti-mouse-fluorescein isothiocyanate (FITC) $F(ab')_2$. Fluorescence signals were collected in logarithmic mode on a FACScan flow cytometer. Results are expressed as percentage positive cells after subtraction of background negative control values.

†Control antibodies included L243 (HLA-DR), VIM13 (CD14), VPM30 (putative ovine CDw75), and 2A4.5 (an anti-phycoerythrin antibody).

‡NANA, Cells treated with neuraminidase from *Clostridium perfringens* before staining.

§Neuraminidase (from *C. perfringens*) treatment and then incubation in medium for 24 h.

¶Neuraminidase (from *C. perfringens*) treatment and then incubation in medium containing tunicamycin at the given concentration for 24 h before staining.

‖SNA, The effect of neuraminidase and tunicamycin treatments were monitored using *Sambucus nigra* (SNA) staining.

recognizes α2,6-linked sialic acid. Tunicamycin in the culture medium prevented re-expression of the structures recognized by CDw75.2–CDw75.5. These findings suggest that CDw75.2–CDw75.5 recognize N-linked glycosylation products and contain sialic acid. In contrast, CDw75.1 binding was revealed or increased by neuraminidase treatment suggesting that the structure recognized by CDw75.1 is at least partially masked by sialic acid. Incubation of neuraminidase-treated PNT cells in tunicamycin did not prevent re-sialylation of the CDw75.1 structure suggesting that the associated glycosylation may not be N-linked.

The reactivity of the CD76 antibodies was extremely heterogeneous on the Namalwa lines. CD76.1 showed moderate→high binding to PNT only; CD76.2 showed moderate binding to G4, IPN/45 and KN2 and moderate→high binding to PNT; B058 (BL-OFX/B8) exhibited low→moderate binding with PNT only; and B057 (8A7) displayed high binding to G4, CSN/70, and PNT and low→moderate binding to NK and IPN/45. ('Unknown' B-cell Panel mAb B057 and B058 were thought to show a pattern of reactivity similar to that of CD76 mAb and were then submitted to the CD76 Panel for testing.) Neuraminidase treatment increased binding of CD76.2 on G4, NK, CSN/70 (and PNT in some experiments), increased B058 binding on all the Namalwa lines, and did not affect binding of B057.

All the CD76 antibodies displayed some binding to the PNT line; B058 exhibited lower binding than the other antibodies. PNT was therefore selected for the neuraminidase and tunicamycin experiments. CD76.1 reactivity was abolished on PNT by neuraminidase and partially recovered overnight in culture (Table 1). This recovery was prevented by tunicamycin. In most experiments CD76.2 was not greatly affected by neuraminidase treatment. An increase in binding of CD76.2 after neuraminidase treatment was noted in some experiments which returned to baseline after overnight culture. Reduced binding of CD76.2 occurred after incubation with tunicamycin. B058 was revealed by neuraminidase treatment and was not remasked during incubation with tunicamycin. If dimethylsulfoxide (DMSO) was used as a vehicle for tunicamycin remasking of B058 occurred. B057 reactivity was slightly reduced after neuraminidase treatment but did not recover overnight in culture and showed a progressive decrease after incubation in increasing concentrations of tunicamycin. These results suggest that the CD76.1-associated glycosylation involves sialic acid residues and is probably N-linked. Some experiments indicated that CD76.2 expression increased after neuraminidase. The CD76.2 structure may therefore be at least partially masked by sialic acid and associated with N-linked glycosylation. The epitope recognized by B058 on PNT is masked by sialic acid and this structure is probably dependent on N-linked glycosylation. The B057 structure may be at least partially sialic acid-dependent and N-linked.

This study indicates the similarity between CDw75.2, CDw75.3, CDw75.4, and CDw75.5 binding following neuraminidase and tunicamycin treatment. CDw75.1 appears to be a different but possibly related specificity. The extreme heterogeneity in the binding pattern of the CD76 antibodies following neuraminidase and tunicamycin treatment was confirmed.

Acknowledgement

J. Andrew is supported by an MRC studentship.

Reference

1. Bast, B. J., Zhou, L. J., Freeman, G. J., Colley, K. J., Ernst, T. J., Munro, J. M., and Tedder, T. F. *J. Cell Biol.* **116**, 423 (1992).

Immunology 1991 **74** 206–214

Expression of the CDw75 (β-galactoside α 2,6-sialyltransferase) antigen on normal blood cells and in B-cell chronic lymphocytic leukaemia

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SUMMARY

Using monoclonal antibodies (mAb) characterized at the last International Workshop on Human Leucocyte Antigens, we examined the expression of CDw75 antigens (β-galactoside α 2,6-sialyltransferase) on normal peripheral blood cells and on cells from patients with B-cell chronic lymphocytic leukaemia (CLL). The mAb used (HH2, EBU.65, EBU.141 and OKB4) detect different epitopes of CDw75. Normal peripheral blood B cells expressed high levels of CDw75 detectable with HH2, EBU.65 and EBU.141 but did not react with OKB4. Cells from patients with B-cell CLL closely resembled normal B cells. All CDw75 epitopes, including OKB4, were strongly expressed on some Namalwa variant Burkitt lymphoma cell lines. The OKB4 epitope was also present on red cells from all normal donors. The other CDw75 mAb were unreactive with red cells from some normal donors. The CDw75 epitope detected with EBU.65 was present on most $CD4^+$ T cells and on a minority of $CD8^+$ cells. HH2 and EBU.141 stained only small numbers of T lymphocytes. OKB4 did not react with T cells. $EBU.65^+$, $CD4^+$ T cells had low levels of expression of CD45R0, CD29, CD54 and CD58, and had high levels of CD45RA antigen. Phytohaemagglutinin (PHA) activation of cells led to the loss of EBU.65 binding. These results suggest that the CDw75 epitope recognized by the EBU.65 mAb is a marker of naive T lymphocytes. On B CLL cells the epitopes detected with HH2, EBU.65 and EBU.141 were destroyed by treatment with neuraminidase. Treatment of B-CLL cells and red cells with neuraminidase increased the binding of OKB4, suggesting that this epitope is masked by sialic acid. The results suggest that CDw75 is a sialylated cell-surface antigen expressed in a number of tissue-specific isoforms.

INTRODUCTION

CDw75 was characterized at the 4th Leucocyte Typing Workshop as an antigen predominantly expressed on B lymphocytes.[1] Four monoclonal antibodies (mAb) comprising CDw75, and a further CDw75-like mAb (EBU.65), had a characteristic staining pattern in immunohistochemistry and reacted strongly with germinal centre B cells and with a subset of cells at the periphery of the germinal centre dark zone proximal to the follicular mantle.[2] The biochemical characteristics of CDw75 antigens, like their function, have yet to be described. However, the CDw75 gene had been cloned and shown to be identical in nucleotide sequence to a previously identified human β-galactoside α 2,6-sialyltransferase.[3,4] The homologous gene in other species encodes liver and kidney enzymes, expressed predominantly in the Golgi apparatus.[5] β-galactoside α 2,6-sialyltransferase is one of a number of structurally unrelated enzymes which catalyse the addition of sialic acid to growing carbohydrate chains of glycoproteins. Carbohydrate structures on leucocyte differentiation antigens appear to be important in a number of aspects of lymphocyte physiology. For example, they serve as ligands for cell adhesion molecules.[6] Tissue-specific and developmentally regulated expression of carbohydrate moieties in non-lymphoid tissues have also been described.[7] There are also differences in sialic acid composition among normal and neoplastic tissues.[8]

Studies at the 4th International Leucocyte Typing Workshop showed that CDw75 is a major component of the lymphoid cell surface[1,9] but did not fully resolve the tissue distribution of the antigen. In view of the possibility that the CDw75 gene encodes a functional α 2,6-sialyltransferase expressed as a lymphoid ecto-enzyme, the aim of the present study was to determine the characteristics of expression of CDw75 antigens in peripheral blood cells.

Abbreviations: BL, Burkitt lymphoma; CLL, B-cell chronic lymphocytic leukaemia; FITC, fluorescein isothiocyanate; mAb, monoclonal antibodies; PBL, peripheral blood lymphocytes; PHA, phytohaemagglutinin; RBC, red blood cells.

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Table 1. mAb used and their sources

Antigen	mAb	Source
CDw75	OKB4	Dr Rao, Ortho, Raritan, NJ
CDw75	HH2	Dr Funderud
CDw75	EBU.141	Dr Gramatzki
CDw75-related	EBU.65	Dr Gramatzki
CD2	RFT11	Scottish Ab Production Unit
CD3	IOT3	Serotech, Bicester, U.K.
CD4	MT310	Dako, Glostrup, Denmark
CD8	DK25	Dako
CD14	VIM13	4th Leucocyte Typing Workshop
CD19	BU-12	3rd Leucocyte Typing Workshop
CD25	Ta60a	3rd Leucocyte Typing Workshop
CD29	4B4	Coulter, Luton, U.K.
CD44	BU52	Binding Site, Birmingham U.K.
CD45RA	F8-11-13	3rd Leucocyte Typing Workshop
CD45RO	UCHL1	Dr Beverley
CD54	84H 10	Serotech
CD58	AICD58	Serotech
CD71	—	Becton-Dickinson, Mountain View, CA
AFP	—	Dr van Heyningen
HLA-DR	L243	American Tissue Culture Collection, Rockville, MD
Activation	4F2	American Tissue Culture Collection

MATERIALS AND METHODS

Monoclonal antibodies

The EBU, HH2 and OKB4 reagents were generous gifts from the originators of the clones. Other mAb were obtained from a variety of sources (Table 1). Antibodies were titrated to determine optimal conditions for use in flow cytometry.

Cell-surface antigen expression analysed by flow cytometry

Only highly viable cell populations (routinely >95%) were examined. Indirect single- and 2-colour immunofluorescence tests were performed by standard techniques. Briefly, in single-colour tests, antibody binding was detected with $F(ab')_2$ anti-mouse Ig conjugated with FITC (Sigma, Poole, U.K.). In 2-colour tests, IgG mAb were labelled with biotin–anti-mouse IgG and streptavidin–phycoerythrin (Amersham International, Amersham, U.K.); all CDw75 mAb are of IgM isotype and were detected with FITC–anti-mouse IgM (Sigma). This reagent was absorbed with IgM-positive human Burkitt lymphoma cells to remove anti-human Ig reactivity, and minimal residual binding of the antibody to human cells was blocked in the test by the addition of 0·01% normal human serum. Cells were suspended and washed in phosphate-buffered saline (PBS) with 1% bovine serum albumin (BSA; Sigma), pH 7·2. All fluorochrome-conjugated reagents were titrated to determine optimal conditions for use. IgG1 and IgG2a anti-alphafetoprotein mAb and a CD14 IgM mAb were included in all tests as isotype-matched negative controls. Flow cytometric analysis was performed with a benchtop flow cytometer (FACScan, Becton-Dickinson, Mountain View, CA) equipped with an argon-ion laser emitting light at a fixed wavelength of 488 nm. Light scatter signals were collected in linear and fluorescence output in logarithmic modes.

Cells and cell culture

The Namalwa panel of variant sublines comprises DNA-fingerprint identical variants of the original Namalwa cell line. The characteristics of the lines have been described in detail;[10] they were cultured in RPMI-1640 medium containing 10% foetal calf serum (FCS) in an atmosphere of 5% CO_2 in air. Blood samples from CLL patients and normal donors were centrifuged over cushions of Histopaque (Sigma) and leucocytes recovered from the interface. Normal peripheral blood cells (PBL) were obtained from laboratory staff and from leucocyte concentrates prepared from normal blood donors. Adherent cells were removed prior to immunofluorescence analysis by incubating cells in plastic tissue culture flasks for 1 hr at 37° in RPMI-1640 medium and 10% FCS. For activation studies, normal PBL were incubated at 37° with 100 μg/ml phytohaem-agglutinin (PHA; Wellcome, Beckenham, U.K.) in RPMI-1640 and 10% FCS, and harvested at times shown.

For neuraminidase treatment, CLL lymphocytes were washed twice in isotonic saline and 10^7 cells were incubated at 37° for 30 min with type X (*C. perfringens*) neuraminidase (Sigma) at a final concentration of 0·05 U/ml. Viability of lymphocytes was unaffected by the neuraminidase treatment. The amount of neuraminidase used was determined by results of preliminary tests.

Haemagglutination and RBC radiobinding assays

For haemagglutination tests, red blood cells (RBC) were washed several times in isotonic saline and the buffy coat cells removed. 0·1 ml aliquots of mAb were added to 0·1 ml of 5% RBC suspensions in saline. After 1 hr at 20° the cells were centrifuged at 200 ***g*** for 2 min, very gently resuspended and examined for agglutination. Scores of 5 (complete agglutination) to 1 (some small clumps of cells) were assigned.

Radiobinding assays were carried out in U-well microtitre plates. 0·05 ml aliquots of 5% washed RBC and mAb were incubated for 1 hr at 20°. Cells were washed twice in PBS-BSA 1%, pH 7·2, and 0·05 ml of ^{125}I-anti-mouse IgM (DuPont Ltd, Stevenage, U.K.) was added (~80,000 c.p.m./well added; specific activity of anti-IgM, ~50 MBq/ml). After a further 30 min at 20°, RBC were washed twice, transferred to fresh tubes and counted for bound anti-IgM. Kendall's test for rankable scores was used to analyse the results of radiobinding assays.

RESULTS

Expression of CDw75 on normal PBL

Peripheral blood samples from laboratory staff and blood donors were tested by indirect immunofluorescence using a panel of CDw75 antibodies and other markers specific for T and B cells. OKB4, HH2 and EBU.141 are mAb assigned to CDw75 at the 4th Leucocyte Typing Workshop.[1] EBU.65 is a mAb which appears to be closely related in specificity to CDw75 reagents.[2,9,11] EBU.65 was in the same preliminary statistical cluster as the other mAb but was not included in CDw75. However, it seems very likely that EBU.65 also belongs in CDw75: in studies as part of the 4th Workshop,[9,11] and in subsequent tests, with Namalwa variant sublines and cells from patients with B-cell malignancy (*v.i.* and also unpublished results), EBU.65 gave 'CDw75-like' staining patterns. Moreover, EBU.141, HH2, OKB4 and EBU.65 all react with COS

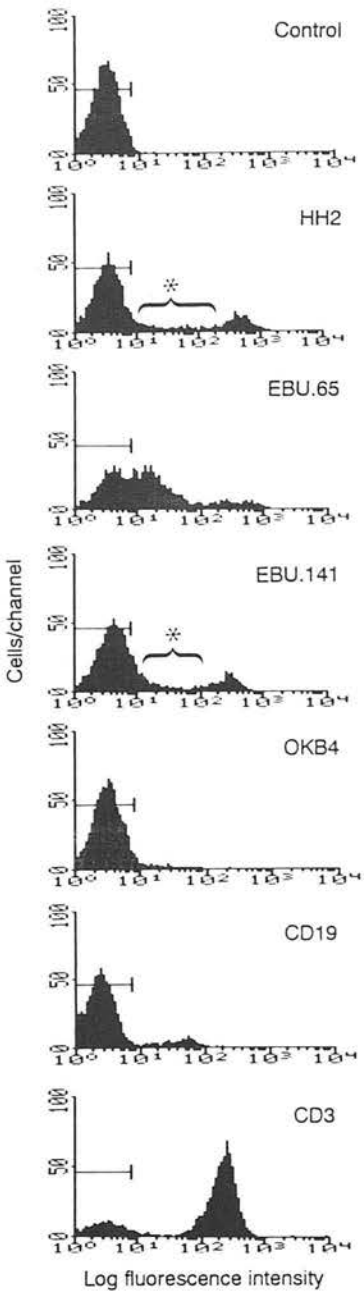

Figure 1. Single-colour immunofluorescence: representative flow cytometry histograms from a series of tests using peripheral blood cells from 12 normal donors. Fluorescence intensity is on a logarithmic and cells/channel on a linear scale. Limits of negative controls indicated by the horizontal bar. Areas of HH2 and EBU.141 histograms marked by asterisks indicate weakly staining cells.

cells transfected with the CDw75 gene (I. Stamenkovic, personal communication).[3]

The staining patterns of PBL from 12 normal individuals were quite similar, and typical flow cytometry histograms for cells from one individual are shown in Fig. 1. Antigens detected by HH2 and EBU.141 were expressed at a high level on a minor population of cells and about a further 5–10% of cells were weakly stained. EBU.141 (mean of $19 \pm 6\%$ positive cells) usually stained a few more cells than HH2 ($14 \pm 5\%$). EBU.65

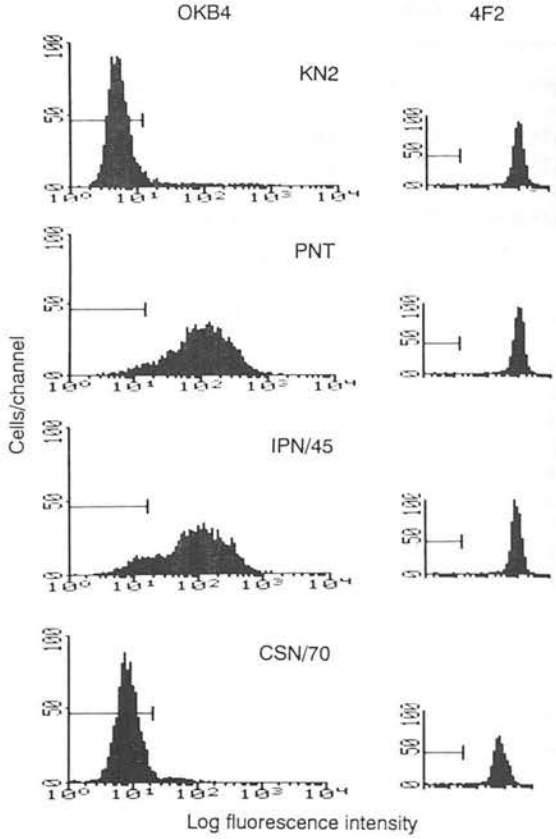

Figure 2. OKB4 tested against Namalwa variant sublines by single-colour immunofluorescence using. Representative results from one of six tests are shown. Other CDw75 mAb were also tested against Namalwa cells with similar results. 4F2 is a mAb to an activation antigen expressed at high levels on cell lines and was included as a positive control. Negative control (limits indicated by the horizontal bar) was the CD14 mAb VIM13 (IgM isotype-matched control).

reacted with many more cells ($51 \pm 7\%$) than the other mAb. Like EBU.141 and HH2, EBU.65 reacted strongly with a minor population of cells and about a further 40% of cells were weakly stained. The total numbers of cells positive with one or other antibody varied to some extent from individual to individual, but the pattern of staining of cells shown in Fig. 1 was evident in all samples tested. In contrast to the other CDw75 mAb, OKB4 consistently failed to react with a significant number of cells ($2 \pm 1\%$) in almost all PBL studied.

CDw75 is differentially expressed on Namalwa BL variant sublines

In a concurrent series of tests using Namalwa Burkitt lymphoma cell lines, OKB4 gave high levels of fluorescent staining (Fig. 2), and in titration tests antibody binding was readily detectable at a dilution of greater than 1 in 10,000. The failure of OKB4 to react with PBL was clearly not an effect due to, say, low titre of the mAb.

Of the panel of Namalwa BL variant cells only two of the four sublines (PNT and IPN/45) had substantial levels of CDw75 expression detectable with OKB4. This differential staining of Namalwa variant sublines was seen in a number of

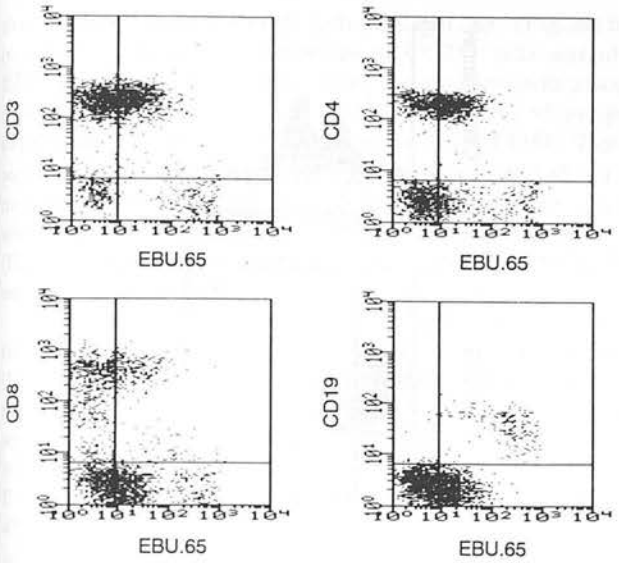

Figure 3. PBL: 2-colour immunofluorescence using FITC-anti-mouse IgM for the detection of EBU.65 (horizontal scale) and biotin-anti-mouse IgG and streptavidin–phycoerythrin for other mAb (vertical scale). Fluorescence intensities are on log scales. Limits of isotype-matched negative controls indicated by the horizontal and vertical lines. Representative results are shown using cells from one donor in a series of tests with cells from nine different normals.

tests performed over a period of about a year. The other CDw75 mAb gave similiar staining patterns, although the reactions of EBU.65 were usually weaker on Namalwa cells than those of the other mAb. We have previously suggested that Namalwa variant sublines are analogues of B cells in different states of cellular maturity.[10] CDw75 may be expressed at the mid stages of differentiation.[1]

Binding of CDw75 mAb to subpopulations of PBL

Normal PBL from nine donors were tested by 2-colour immunfluorescence, with CDw75 mAb and specific markers for B or T cells. Representative results using EBU.65 are shown in Fig. 3. The small population of strongly-stained cells with EBU.65, EBU.141 and HH2 were CD19$^+$ B cells. All B cells were CDw75$^+$. CDw75 was also expressed on T cells: the cells with a low level of EBU.65 expression were mostly CD4$^+$ T cells and some CD8$^+$ cells were also EBU.65$^+$. There was considerable variation in the numbers of EBU.65$^+$ T cells among different donors: the numbers of EBU.65$^+$ cells among CD2$^+$ or CD3$^+$ T cells ranged from 33% to 52%. The range of expression of EBU.65$^+$ cells among CD4$^+$ T cells was 38–75%. Smaller proportions of CD8$^+$ cells (range 8–34%) were EBU.65$^+$. Usually CD8$^+$, EBU.65$^+$ T cells had high levels of CD8 expression. Only small numbers of T cells (a mean of 7% CD2$^+$ cells) were stained with EBU.141 or HH2. Most of these were CD4$^+$ and only 2–3% of CD8$^+$ T cells were routinely EBU.141$^+$ or HH2$^+$ (data not shown).

CDw75 and CD45R expression on lymphocytes

The possibility that the expression of CDw75 antigens on subpopulations of T cells is a reflection of differences in the maturity and/or state of activation of the cells was investigated.

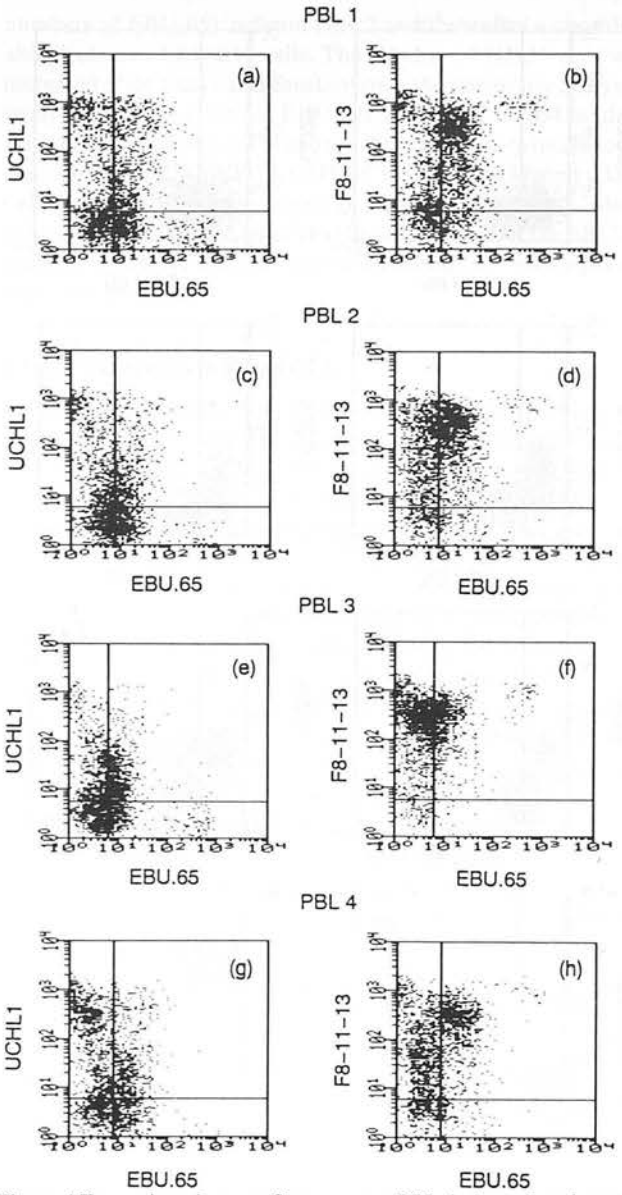

Figure 4. Two-colour immunofluorescence: PBL 1–4 are lymphocytes from four different normal donors. Presentation of results as in Fig. 3. UCHL1 is a CD45RO and F8-11-13 a CD45RA mAb.

In the first instance the expression of leucocyte common (CD45) antigens and CDw75 antigens was compared (Fig. 4). CD45 antigens were detected with UCHL1 (CD45RO) and F8-11-13 (CD45RA) mAb. The expression of CD45RA on T cells has been shown to define a population of virgin/immature or naive cells, while CD45RO$^+$ cells comprise the memory T-cell population.[12]

There was considerable variation in the expression of CD45 antigens on normal PBL from person to person. On cells from three of the four donors shown there was also very considerable heterogeneity in CD45RO and CD45RA expression among cells from the same individual. That is, there were populations of cells with apparently high levels of antigen expression and others with much lower levels of antigen using both UCHL1 and F8-11-13 mAb. From the numbers of positive cells it was clear that some cells expressed both CD45RA and CD45RO. This

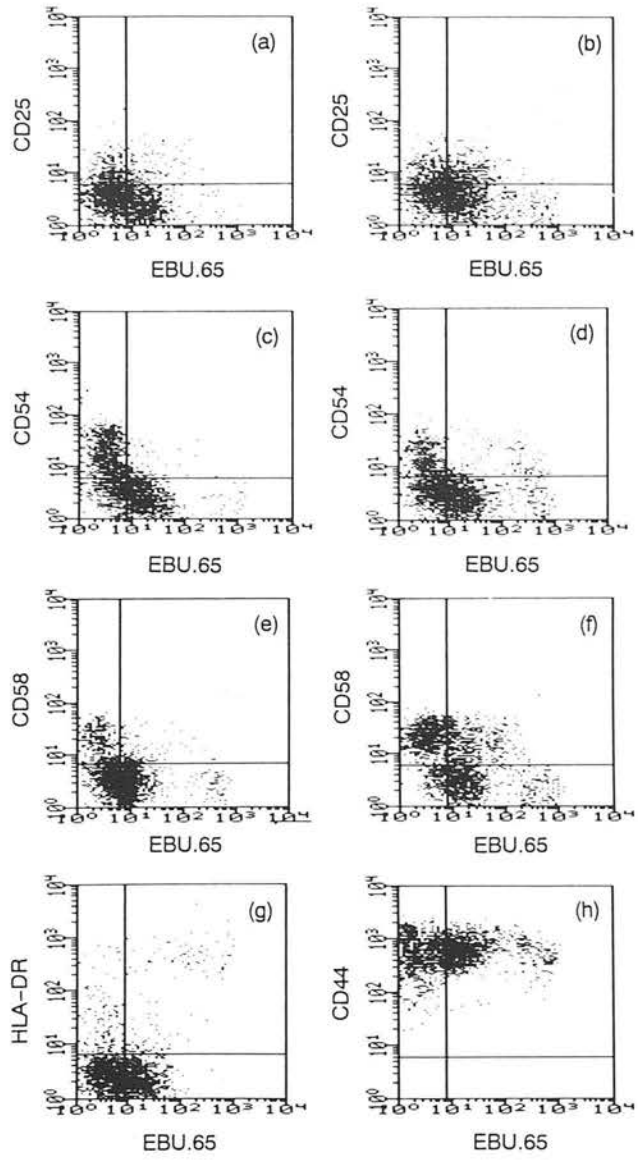

Figure 5. Two-colour immunofluorescence with normal PBL. Representative results from tests with several different normal donors are shown. Presentation of results as in Fig. 3.

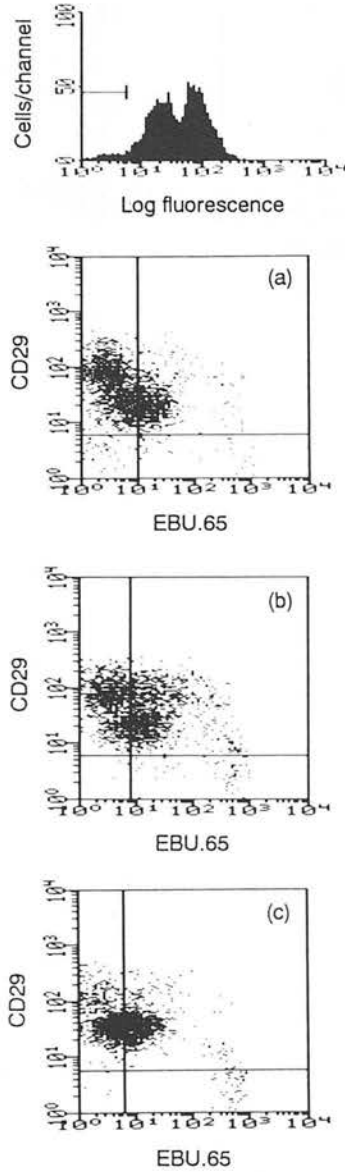

Figure 6. CD29 expression in normal PBL. Results from three different normal donors (a, b, c) are shown. The top histogram shows single-colour (donor a) and the rest 2-colour immunofluorescence. Presentation of results as in other figures.

dual expression of CD45R epitopes has been seen in other studies and may accompany the passage of cells to and from memory T-cell compartments.[12]

In all normal donors, most cells with a low level of CD45RO expression were EBU.65 positive (Fig. 4). Fewer cells in the CD45RO brightly staining fraction were EBU.65 positive. However, the numbers of cells in this brightly staining fraction and their binding of EBU.65 varied a lot from donor to donor. In three of the four normal donors shown, the majority of cells with a high level of CD45RA were EBU.65$^+$. In the other donor (PBL 3), where there were few CD45RA^{dim} and few CD45RO^{bright} cells (Fig. 4e, f), about half of the CD45RA$^+$ cells were EBU.65$^+$. B cells (strongly stained with EBU.65) were CD45RA$^+$and CD45RO$^-$ in all donors. Because of the considerable variation in CD45 expression among normal donors, the heterogeneity among cells from a given donor, and the expression of CDw75 on some cells with a high level of CD45RO expression, the meaning of these results is not straightforward. However, they suggest that—in most normal donors—the expression on T cells of the CDw75 epitope recognized by EBU.65 occurs predominantly on naive T lymphocytes.

CDw75 and activation marker expression

To examine expression of the EBU.65 epitope on T-cell subpopulations in more detail, cells from normal donors were tested with mAb to HLA-DR, CD25 (interleukin-2 receptor), CD29 (VLA integrin β-1 chain), CD54 (LFA-3), CD58 (ICAM-1) and CD44 (Pgp-1). Figure 5 shows representative results from a larger series.

There was no consistent association between EBU.65 binding and CD25 expression among different donors. In some

donors the majority of CD25$^+$ cells were EBU.65$^-$ (Fig. 5a) but in others a significant proportion of CD25$^+$ cells was also EBU.65$^+$ (Fig. 5b). Similarly, there was no consistent association of HLA-DR on T cells (Fig. 5g) and EBU.65 antigen expression. However, in all donors tested, most CD54$^+$ T cells were EBU.65$^-$. Most CD58$^+$ T cells were also EBU.65$^-$. The numbers of CD58$^+$ cells varied from donor to donor and in some a minor population of CD58$^+$ cells was also EBU.65$^+$ (Fig. 5f). There were no consistent differences in CD44 (Fig. 5h) expression among EBU.65$^+$ and EBU.65$^-$ cells.

Using 4B4, a CD29 mAb,[13] there was consistent staining of two populations of cells with clear differences in antigen density (Fig. 6). The number of CD29$^+$ cells (and the proportions of dim and bright cells) was very variable among different donors. In some donors, EBU.65 binding was confined to the dim CD29$^+$ cells. In others some of the brightly stained cells were also EBU.65$^+$. Using a directly conjugated 4B4 preparation (FITC-4B4), high levels of CD29 expression on PBL were found on cells with high levels of CD45RO, CD18, CD54 and CD58 antigens. As well as their patterns of CD45 antigens, memory T cells are characterized by the expression of high levels of CD18, CD29, CD54 and CD58.[14] Together with the findings on CD45R expression, the results suggest that most EBU.65$^+$ T cells belong to a naive T-cell compartment rather than to memory populations.

Changes in CDw75 expression after PHA activation of PBL

Cells from two normal donors stimulated with PHA were tested over a period of a week (Table 2). There was an initial rise in the numbers of EBU.65$^+$ cells on Day 2 and thereafter a considerable decline in EBU.65$^+$ cells. The number of HH2$^+$ cells was increased after 2 days and constant over the remaining 5 days of study. There was a loss of EBU.141$^+$ cells and OKB4 binding was unaffected. By Day 2 of culture there were sharp increases in expression of CD25, CD29, CD54, CD58, HLA-DR and CD71 (transferrin receptor). Analysis of CD4$^+$ T cells by 2-colour immunofluorescence showed that there was a loss of the EBU.65 and a gain of HH2 epitope expression during the culture period (data not shown).

Table 2. Expression of CDw75 and CDw75-related antigens on PHA-stimulated PBL

	% positive cells							
	Donor 1				Donor 2			
mAb	Day 0	Day 2	Day 5	Day 7	Day 0	Day 2	Day 5	Day 7
HH2	9	36	26	29	8	26	29	33
EBU.65	54	72	13	22	43	51	14	20
EBU.141	23	31	10	16	12	18	9	9
OKB4	2	5	2	6	2	5	4	3
CD3	94	99	100	—	87	97	—	100
CD25	25	97	100	96	18	96	99	99
CD45RA	96	100	97	—	82	98	95	86
CD45RO	56	91	99	—	67	90	100	100
CD54	18	97	99	95	34	95	99	98
CD58	29	38	94	91	—	—	—	—
CD71	1	74	93	79	6	74	100	68
HLA-DR	7	75	50	52	7	56	64	56

Cells were tested by indirect immunofluorescence and results are expressed as percentage positive cells. Negative controls were routinely 1% positive cells. Viability of the cells was routinely >95%. Cells not tested indicated by —. Nos 1 and 2 are cells from two normal blood donors. Cells stimulated with 100 μg/ml PHA and harvested on the days shown. Day 0 = unstimulated cells.

CDw75 expression in B-cell CLL

Peripheral blood cells from about 40 CLL patients were tested with CDw75 mAb and other mAb, including CD3, CD19 and CD5. HH2, EBU.141 and EBU.65 gave high levels of staining on all B lymphocytes on cells from nearly all patients (Fig. 7). Cells were also tested after treatment with neuraminidase (*v.i.*). Some T cells in samples from CLL patients were weakly CDw75$^+$. In about 10% of samples from CLL patients there was a low level of OKB4 staining and in the remaining cases OKB4 binding was not detectable. In a very few samples there were also significant differences in the patterns of staining obtained with HH2 and EBU.141 (data not shown)—HH2 sometimes stained only a minor proportion of EBU.141$^+$ and EBU.65$^+$ cells. These atypical results were confirmed by repeat testing of samples obtained from the same patient on several different occasions. Together with the results of PHA activation of cells, these findings suggest that HH2 and EBU.141 mAb recognize different CDw75 epitopes. Indeed, the CDw75 mAb tested in this study appear to be directed to four different epitopes.

Sialic acid is a component of CDw75 epitopes

Cells from patients with CLL were also tested after neuraminidase treatment. A previous report showed that neuraminidase treatment destroyed the epitope recognized by the LN1 CDw75 mAb.[15] In about 20 cases tested, the binding of HH2, EBU.141 and EBU.65 was completely ablated by neuraminidase treatment of the cells (Fig. 7). In a further three cases there was a partial reduction in HH2, EBU.141 and EBU.65 binding. In contrast, on the same panel of cells the binding of OKB4 was increased substantially, in almost all cases, by neuraminidase treatment. The binding of anti-HLA-DR, CD19 and CD2 mAb to CLL cells was unaffected by neuraminidase. These results suggest that the HH2, EBU.141 and EBU.65 epitopes comprise or depend structurally upon, sialic acid residues. The CDw75 OKB4 epitope is shielded from antibody binding by sialic acid.

CDw75 epitopes are expressed on RBC

A total of 23 samples of red cells from normal donors and from CLL patients was tested initially by haemagglutination. OKB4 produced complete or strong agglutination in all cases. Not all samples were so strongly reactive with the other mAb, and using EBU.65 there was no detectable haemagglutination with more than half of the RBC samples tested. Haemagglutination with OKB4 was readily detectable at dilutions of more than 1/25,000. Neuraminidase treatment of RBC increased the titre of OKB4 and abolished agglutination produced by HH2, EBU.65 and

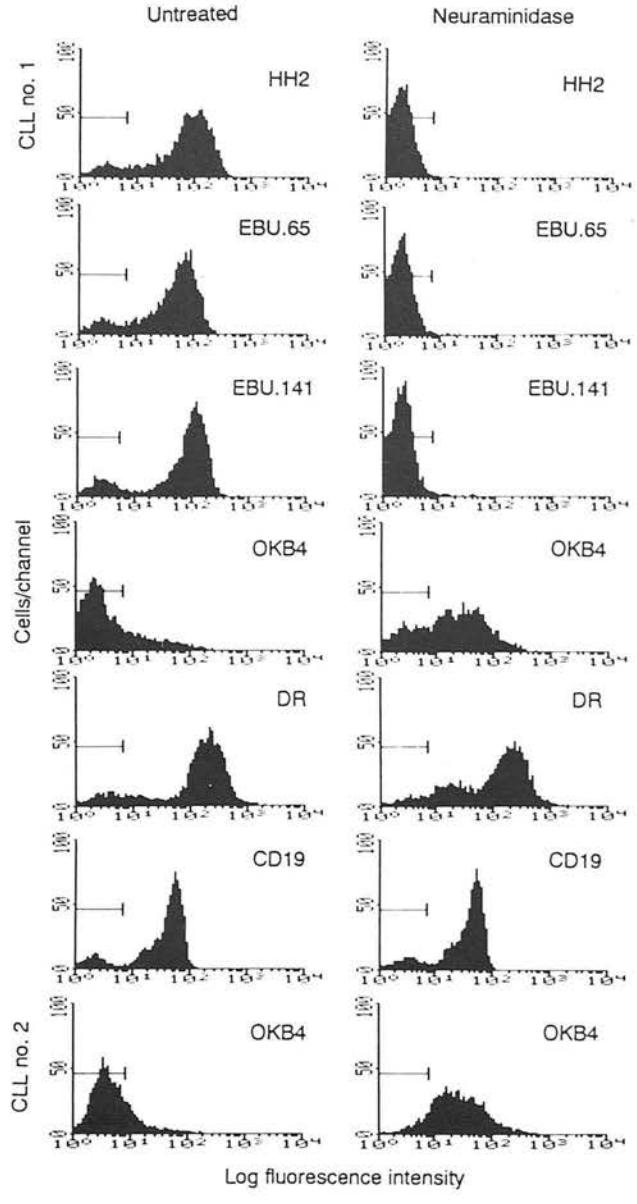

Figure 7. CDw75 expression in CLL patients. Single-colour immunofluorescence: representative flow cytometry histograms using cells from one CLL patient (CLL no. 1) from a series of more than 40 different patients' samples are shown. Results presented as in Fig. 1. Cells shown as untreated samples and after incubation with neuraminidase. Anti-HLA-DR and CD19 results are included as negative controls for neuraminidase treatment. CD2 was similarly unaffected by neuraminidase treatment. Also shown are cells from a second CLL patient (CLL no. 2) tested with OKB4.

EBU.141 (data not shown). In further tests using cells from a normal donor panel typed for common blood group antigens, both haemagglutination and a radiobinding assay were used. By radiobinding assay, OKB4 binding was readily detectable on all samples tested (Table 3). EBU.65 binding was weak or negligible on more than half of the same samples. There was also only a low level of EBU.141 and HH2 binding on RBC from several donors. With one exception (OKB4 versus EBU.65) there were

Table 3. Reactions of CDw75 mAb with typed red cell panel from normal donors

Donor no.	c.p.m. ^{125}I-AMIgM bound			
	OKB4	EBU.65	EBU.141	HH2
1	5539 (5)	262 (1)	2309 (3)	6321 (4)
2	9000 (5)	1162 (1)	5272 (4)	9116 (4)
3	4414 (4)	306 (1)	2782 (4)	4123 (3)
4	8383 (5)	2704 (4)	8739 (5)	11417 (4)
5	4961 (4)	191 (0)	559 (1)	1751 (2)
6	7512 (4)	37 (0)	1336 (2)	4142 (3)
7	4309 (4)	244 (1)	2027 (2)	3825 (3)
8	4158 (5)	153 (0)	505 (1)	1875 (1)
9	6833 (5)	925 (2)	4430 (4)	7930 (4)
10	7499 (5)	892 (4)	3436 (4)	7597 (4)

Binding detected with ^{125}I-anti-mouse IgM (duplicate wells). Results expressed as mean c.p.m. bound after subtraction of binding of ^{125}I-AMIg to cells measured in the presence of an irrelevant IgM mAb (background binding values 130–476 c.p.m.). Numbers in parentheses are the results of haemagglutination tests: (5) = complete agglutination and (0) = no detectable agglutination.

highly significant positive correlations in the binding of the different mAb to RBC.

Leucocytes from the panel of RBC donors tested were not available but on other normals and on cells from CLL patients there were high levels of expression of EBU.65, EBU.141 and HH2 antigens on lymphocytes from donors whose RBC were weak or negative for these antigens. It is a striking finding that the expression of the different CDw75 mAb to RBC is the reverse of their appearance on normal lymphocytes: that is, apparently high levels of OKB4 and low levels of EBU.65 binding are found on RBC, while the opposite is true for PBL. The panel of normal RBC donors used had been typed for ABO, Rhesus, M, N, S, s, P1, Le^a, Le^b, Lu^a, Lu^b, K, k, Fy^a, Fy^b, Jk^a, Jk^b, Xg^a and Co^b antigens. The specificity of CDw75 mAb did not correspond to any of the polymorphic blood group antigens.

DISCUSSION

B-galactoside α 2,6-sialyltransferase is an enzyme previously characterized in the rat as a component of the Golgi apparatus in liver cells.[5,6] To our knowledge, in non-human species it has not been recognized as a molecule expressed in lymphoid tissues. Studies for the 4th International Workshop on Human Leucocyte Differentiation Antigens established the antigen recognized by CDw75 mAb as a major component of the lymphoid cell surface.[16] Subsequently, using the HH2 mAb, the CDw75 gene was cloned after cDNA transfection in COS cells,[3] and shown to be homologous in nucleotide sequence to the rat α 2,6-sialyltransferase gene. In human lymphoid cells the function of the CDw75 antigen is not known; if the molecule has sialyltransferase activity it might be expected to have a significant impact on the glycosylation patterns of cell surface molecules. In this way CDw75 could be influential in determining the course of lymphoid differentiation. There remains the question of why

α 2,6-sialyltransferase should be expressed as an ecto-enzyme. It has been suggested that the enzymatic activity of some glycosyltransferases could be redundant and they might have other functions—for example, in cell adherence—but as yet this is unresolved. The function of α 2,6-sialyltransferase has been explored in a developmental system: in *Xenopus* embryos injected with mRNA encoding α 2,6 sialyltransferase, polysialylation of the neural cell adhesion molecule (N-CAM) is prevented and abnormalities of neural development result.[17]

The principal finding of the present study is that a number of different isoforms of CDw75 exist and these are detectable by differences in antibody binding. Several isoforms occur simultaneously in B cells, whereas subpopulations of T cells have a more restricted scheme of expression of the different CDw75 epitopes. B cells also have very much higher levels of CDw75 expression than T cells. Sialic acid is a component of the epitopes recognized by CDw75 mAb since binding to red cells and leucocytes of three of the four mAb used can be abolished by treating cells with neuraminidase. The epitope recognized by the remaining mAb (OKB4) appears to be masked by sialic acid and is revealed on neuraminidase-treated cells. The different isoforms of CDw75 might be created by additional glycosylation of a core carbohydrate sequence.[18] The masking of epitopes by sialic acid is a feature of other antigens. For example, epitopes of the Xg blood group-related MIC2 gene product are shielded from antibody binding on red cells by sialic acid.[19] Treatment of acute myeloid leukaemia cells with neuraminidase reveals the carbohydrate CD15 antigen, 3-fucosyl-N-acetyllactosamine.[20] Epitope shielding could be a common phenomenon which might restrict cellular interactions involving sialic acid-containing carbohydrate moieties on glycoproteins. For CDw75, the presence of sialic acid as a component of a molecule which is a sialyltransferase is intriguing and raises the question of whether or not CDw75 is a target for additional lymphoid sialyltransferases.

So far there is little information on the biochemical characteristics of antigens recognized by CDw75 mAb, despite the fact that some of the mAb have been available for several years.[15,21–23] In contrast, in the rat, α 2,6-sialyltransferase has been purified to homogeneity.[24] In a series of biochemical studies, using a wide variety of detergents, we have had great difficulty in retaining antigenicity of CDw75 after solubilization of cells (J. M. Andrew, unpublished results). Biochemical studies as part of the 4th Leucocyte Typing Workshop also failed to determine the characteristics of antigens recognized by CDw75 mAb. This is a considerable block to defining the precise tissue distribution of CDw75. Antibodies directed to carbohydrate epitopes on glycoproteins are obviously unreliable for tracking the expression of the corresponding polypeptide, because of possible differences in the glycosylation of the molecule in different tissues. This is a particular difficulty in following the tissue distribution of human lymphoid α 2,6 sialyltransferase with the available CDw75 mAb. Although only limited data are available, the tissue distribution of CDw75 has been examined by Northern blotting[3] and levels of mRNA did appear to correlate with the expected levels of CDw75 cell-surface antigen expressed on peripheral blood B and T cells. It is possible that differential glycosylation alone accounts for the apparent variations in structure of CDw75 on lymphocytes and red cells. However, there is a potential for significant heterogeneity in polypeptide structure: in the rat, the α 2,6-sialyltransferase gene specifies divergent polypeptides in liver and kidney.[25] This is achieved by alternative splicing mechanisms to generate more than one kidney enzyme and by differences in transcriptional initiation sites to generate different kidney and liver polypeptides.

Prior to their assignment to a specific cluster, CDw75 mAb were tested against leucocytes in a number of studies and OKB4 was shown to react with the majority of normal peripheral blood B cells.[23] However, in the present study we have been unable to demonstrate this level of reactivity. The reasons for this discrepancy are not clear since the OKB4 mAb we have used is of high titre and reacts with high fluorescence intensity on BL cell lines and, even when used at very high dilutions, it completely agglutinates red cells. In studies for the 4th Leucocyte Typing Workshop the consensus was that OKB4 reacted with about 30% of peripheral blood B cells.[16] These differences in results might in part be explained by the finding in the present study that the OKB4 epitope on B cells is masked by sialic acid residues.

The relationship of epitopes on leucocyte CD antigens to blood group antigens on red cells is an area for further study. For example, the 3-fucosyl-N-acetyllactosamine epitope [also known as X-hapten and stage-specific embryonic antigen (SSEA-1)] recognized by the CD15 mAb is formed in myeloid cells by the action of a chromosome 11-encoded α 3-fucosyltransferase.[26] The Lewis blood group fucosyltransferase (an α(1,3/1,4)fucosyltransferase), which maps to chromosome 19, is also capable of synthesizing the SSEA-1 epitope.[27] Some blood group antigens (In^a and In^b antigens) are present on leucocyte and erythrocyte CD44 molecules.[28] The differential staining of leucocytes and red cells from some normal subjects by CDw75 mAb is rather like the reactions of mAb against some epitopes of the CD44 glycoprotein: the In^b blood group antigen on CD44 molecules is absent (or reduced in expression) from red cells in some individuals whose leucocytes are positive. While lack of expression of the EBU.65 epitope on red cells appears to be a relatively common polymorphism, in the case of CD44 it is the rare dominant form of Lutheran blood group-negative donors who are lacking in red cell In^b antigens. In^b expression on red cells is specifically influenced by the action of a dominant inhibitor gene called *In(Lu)* which affects red cell expression of a number of high frequency blood group antigens.[29]

The tissue specificity of CDw75 mAb is likely to be determined by the specific pattern of glycosylation of the molecule in different tissues. Therefore, it may be necessary to prepare mAb against polypeptide determinants to fully appreciate the distribution of α 2,6-sialyltransferase in human cells. The differences in the tissue distribution of CDw75 epitopes reported herein is an indication of the likely importance of carbohydrate moieties to the micro-heterogeneity of leucocyte differentiation antigens. This may extend to modifications of the functional properties of these molecules. A detailed analysis of the structural relationships among products of the α 2,6-sialyltransferase gene expressed in different tissues should help in understanding the function of the CDw75 molecule in lymphocytes.

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B009 (6C12), B028 (J3-6), B049 (1D8), B058 (BL-OFX/B8), and B064 (B5) were limited to the FAB-M4 or -M5, and positive tests with B015 (CMRF-41), B027 (J3-89), and B055 (HB-4) were observed only with FAB-M1, -M2, or -M3 subtypes. mAb B011 (EBU-1166) is of special interest in that it reacted with seven of 11 AML including FAB-M1, -M3, -M4, and -M5 types but not with blasts of B- and T-lineage ALL.

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B19.7 Potential cluster analysis with variant sublines of Namalwa

J. A. ROSS and J. ANDREW

Table 1 Phenotype of Namalwa variant sublines using a panel of mAb

Antibody designation	Percentage positive cells*				
	G4	NK	CSN	PNT	KN2
4F2†	94	97	96	98	98
HLA II‡	23	76	69	99	98
HLA-DR	13	64	65	98	98
HLA-DP	1	10	17	81	89
HLA-DQ	3	15	26	94	85
CD7	82	69	39	5	1
CD9	86	94	97	90	94
CD10	5	35	83	96	59
CD11a	7	20	6	7	11
CD11b	2	10	10	4	2
CD11c	4	14	11	8	2
CD17	18	10	39	3	4
CD18	3	25	8	5	4
CD19	18	59	74	78	16
CD20	8	16	6	22	1
CD21	4	9	26	10	2
CD22	33	30	65	70	5
CD23	13	36	30	20	4
CD24	18	73	92	93	88
CD25	20	26	9	8	84
CD37	32	55	54	91	58
CD38	95	91	92	98	98
CD40	90	82	95	69	45
IgM	–	6	23	93	66
CD43	91	59	74	95	96
CD45	89	95	96	99	98
CD45RO	94	92	81	20	34
CD54	93	97	94	98	98
CD71	67	75	76	84	89

continued

Table 1 *(continued)*

Indirect immunofluorescence using rabbit anti-mouse-FITC $F(ab')_2$. Results are expressed as percentage positive cells after subtraction of background negative control values. Fluorescence signals were collected in logarithmic mode on a FACScan flow cytometer.
†4F2 recognizes an activation antigen on human cells.
‡DA6.231 recognizes a framework structure on MHC class II molecules.

The Workshop B-cell Blind Panel was tested against a series of variant sublines of the Burkitt lymphoma cells Namalwa. The Namalwa variant lines derive from the same tumour biopsy and are DNA fingerprint identical. They have marked differences in Ig heavy-chain and light-chain structure and expression, and have a number of different mutations in the flanking

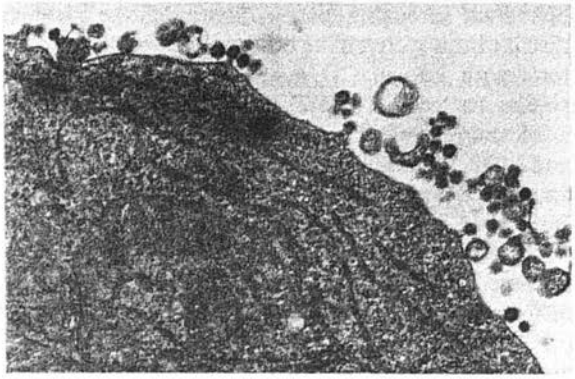

Fig. 1 Portion of a cell from the Burkitt's lymphoma line Namalwa (IPN/45) with mitochondria and rough endoplasmic reticulum in the cytoplasm. A large number of viral particles are seen in the extracellular milieu. Type D retroviruses are seen at various stages of budding from the cell surface membrane to produce virions.

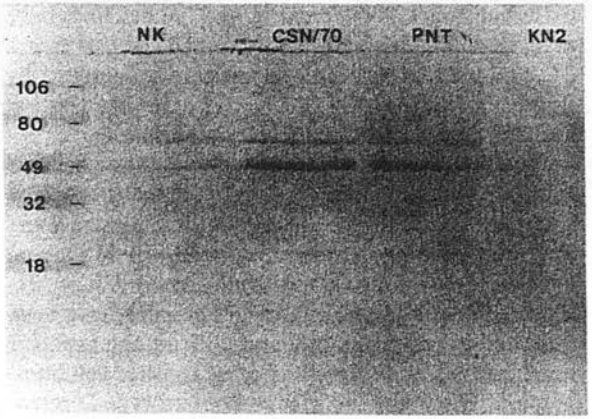

Fig. 2 Western blot of B029 (By2). The structure recognized by B029 appears to be differentially expressed in the Namalwas being greatest on CSN/70 and PNT. Weak bands are seen with NK and bands are almost absent from the KN2 lane. The bands appear to be 64, 47, and a weak band at 18 kDa in all lines. Molecular weight markers are shown.

sequences of the c-*myc* locus [1]. They display non-coordinate expression of a number of cell surface structures. The six sublines in the complete panel are, in increasing order of maturity, G4→NK→CSN/70→IPN/45→PNT→KN2.G4 and NK display some lineage infidelity (for example, CD7 expression), CSN/70 has no surface immunoglobulin (Ig), PNT has surface Ig, and KN2 secretes Ig. The different sublines are phenotypically approximate to B cells arrested at

Table 2 Reactivity of potential clusters with a cell panel including the Namalwa variant sublines

Workshop mAb		Percentage positive cells*								
Code	Clone name	NK	CSN	IPN	PNT	KN2	PBL1	PBL2	B-CLL	Reactivity†
B006	DFB1	34	85	99	99	99	63	74	86	CD45RA
B044	Ki-B5	32	81	99	99	81	36	76	98	CD45RA
B008	B5	53	94	92	98	59	7	6	86	Unclustered
B064	B5	37	95	93	96	59	4	–	87	Unclustered
B012	EBU-65	–	–	67	39	5	33	60	97	Unclustered
B027	J3-89	3	5	60	26	6	29	57	98	Unclustered
B019	M9	99	99	99	99	99	96	94	38	Unclustered
B071	4TM-1	99	99	99	99	99	96	95	10	?PC#4
B031	VMP55	34	75	94	99	97	1	26	36	Unclustered
B032	GHI/75	32	69	92	99	96	17	25	58	Unclustered
B065	HB15a	20	20	–	–	–	4	–	–	PC#3
B066	HB15b	16	23	–	–	–	4	–	–	PC#3
B067	1D6	99	99	99	99	99	97	99	31	PC#4
B068	5A6	99	99	99	99	99	97	95	35	PC#4
B069	JS64	99	99	99	99	99	98	97	54	PC#4
B070	4TM-1	99	99	99	99	99	97	96	67	PC#4

*Indirect immunofluorescence using rabbit anti-mouse fluorescein isothiocyanate (FITC)-conjugated $F(ab')_2$. Results are expressed as percentage of positive cells without subtraction of background negative control values (~3%). Fluorescence signals were collected in logarithmic mode on a FACScan flow cytometer. PBL, peripheral blood cells; B-CLL, B-cell chronic lymphocytic leukaemia. –, ≤Negative controls.
†PC, Preliminary cluster to be assigned at Workshop.